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C.-E. A. Winslow

It is characteristic of Science and Progress that they continually open new fields to our vision.—Pasteur.

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THE INFLUENCE OF H ION CONCENTRATION UPON STRUCTURE

I. H. INFLUENZAE

GUILFORD REED AND J. H. ORR

Queen's University, Kingston, Canada

Received for publication May 28, 1922

Most observers of Pfeiffer's bacillus make reference to its structural variability. Coccoid or filamentous forms have frequently been found, along with, or replacing, the typical small rods.

Ritchie (1910) isolated an atypical form from spinal fluid and Wollstein (1915) concluded that the species could be divided into long forms and short forms while Bell (1920) more recently separated three forms which he finds after long cultivation to remain true to type. Certain of Bell's types however suddenly changed to long filaments and as suddenly changed back to the typical form; no explanation of the phenomenon is offered. Lacy (1918) and Maitland and Cameron (1921) have reported the isolation of rod and filamentous forms from the same case.

More distinctly germane to the present discussion are the observations of Valerio (1919) who found ovoid, spherical, clubbed and filamentous forms, especially in old cultures, and those of Wade and Manalang (1920) who found distinctly fungoid forms in cultures of *H. influenzae*. The latter workers report that "under certain conditions" very long filaments with various irregular swellings which they regard as reproductive bodies are developed. These forms appeared in a beef infusion -peptone-salt-boullion containing laked sheeps blood and (what is particularly interesting in view of the following discussion) adjusted "to about 1 per cent acid" and sterilized by filtration or by heat at a low temperature.

During the last four years a number of strains of *H. influenzae* have been under cultivation in this laboratory. These have been isolated for the most part from epidemic cases, some from various pathological conditions when no epidemic influenza was known to exist in the community; and several strains have been sent us from other laboratories.[1]

1. These cultures were maintained on a standard medium carefully made so that successive lots were as nearly identical as possible. This was an ordinary beef extract peptone agar made up in every case in the same manner, adjusted with NaOH to pH value of 7.4 and a phosphate mixture of this acidity was added to make the finished medium 0.05M phosphate. The medium was autoclaved and cooled to 80°C., enough whole rabbits blood added to make the finished medium 1 per cent blood, maintained for ten minutes at 80°C. and tubed or plated.

On this medium about half of the strains examined exhibited the conventional rod forms 0.2 to 0.3 by 0.5 to 1.0μ with slightly rounded ends and parallel sides, and a small number of oval or coccoid forms 0.3 to 0.5μ in diameter (plate 1, *1*). Other strains on this standard medium consisted of these uniform short rods along with a few filamentous types 0.2 to 0.5 by 5 to 10μ, generally somewhat curved and frequently tapering at the ends (plate 1, *2*). A small number of strains show the short rods and the filamentous types in about equal numbers. A still smaller number of strains consist predominantly of the filamentous types with only a small number of small rod forms (plate 1, *3*). All of these forms usually stain uniformly although with watery fuchsin a few of the small rods show bipolar granules and frequently the filaments show granules at one end or throughout their length. (See table 1 for a summary of the occurrence of these types in 16 strains.)

Agglutination reactions failed to show any group agglutinins corresponding with the morphological types. Anti-serum was prepared for 11 of the strains, representing the four structural

[1] We are indebted to Prof. E. O. Jordan, Chicago; Dr. W. H. Park, New York; Dr. T. M. Rivers, Baltimore, and Professor H. B. Maitland, Toronto, for one or more cultures.

types and cross agglutinations carried out. These results indicated strains 7 and 10 to possess some relationship; strains 2, 3, 4, 5, and 9 to be somewhat related to each other; and strains 6, 8, 11 and Neg. to be unrelated both to each other and to the other strains. Reference to table 1 will indicate that these strains related by agglutination results belong to different morphological groups.

Where a pure strain shows more than one morphological type the several forms appear in a single colony. Smears made from a number of discrete colonies from various parts of a culture surface are always very similar or identical; any one colony usually shows all the structural types present in the culture and about the same percentage distribution in the colony as in the entire culture.

A conspicuous feature of all these strains is the constancy of the morphological types as long as they are maintained upon this standard medium. Many of these strains have been carried through a large number of cultural generations with very little change from their original form. This is conspicuously true of those strains showing originally the characteristic short rods; these produced a few filamentous forms from time to time but none of them show any tendency to change to a predominantly coccoid or filamentous type. This, however, is only true when the medium remains constant.

2. Although exhibiting a considerable degree of structural stability when grown on a constant medium most of these strains are remarkably pleomorphic on certain other media. Various modifications of the chemical and physical state of the medium resulted in morphological changes, but the most conspicuous and constant results were produced by varying the hydrogen ion concentration of the substratum.

Media prepared like the standard medium, described in the previous section, were buffered with phosphates to a range of H ion concentration from the most acid to the most alkaline which will support the growth of this organism. The same amount of fresh blood (1 per cent) was added to each lot, and the medium was heated to 80°C. for ten minutes and tubed or plated.

A number of strains have from time to time been grown on these media of graded acidities and 16 strains were compared in some detail. These strains were subcultured several times from single discrete colonies, while in 2 cases single organisms, isolated by the Barber method, were made the parents of subsequent generations.

All the strains grown upon media buffered to pH values of about 6.8 to 7.8 present the same structural types as described in the previous section (see table 1), i.e., the acidity of the media may be varied through the above mentioned range without effecting conspicious alterations in the morphology. It is not possible, however, to define accurately the acidity limits of the media for what may be styled normal morphology. Transition from the normal to the abnormal form is a gradual change and moreover the degree of acidity or alkalinity necessary to effect observable modification varies slightly with different strains. The stated acidity limits, however, approximately define the limits of normal structure in the strains studied.

This range of media, pH 6.8 to 7.8 supporting normal morphology at the same time permits very nearly maximum growth of the species. The amount of growth is slightly greater at a point about midway between these limits than at the extremes but the difference is slight. Avery (1918) made his media for maximum growth pH 7.3 to 7.5 and Bell (1920) found pH 7.3 to 7.4 the most desirable reaction.

In acid media, from pH 6.5 to the maximum acidity for growth, all the strains exhibited some structural variation from the normal form and in most strains the change was extreme. The normal forms of the neutral range of media were replaced in part, in some cases entirely, by long filaments, by various coccus-like or yeast-like forms or by a variety of swollen and irregular shaped forms. (See table 1 for a summary of the occurrence of types.)

The filamentous forms range in length from 5 to 50 microns and occasionally to 100 microns by 0.2 to 1 micron; usually they lie in an undulating wave-like position, some are sharply bent, the ends generally are pointed, occasionally square or rounded.

In a few instances branching has been observed. In most strains a considerable number of the filamentous forms show globoid or irregularily oval swellings at the end or along the course of the filament. The filaments, especially the larger ones, stain rather more deeply with watery fuchsin than the normal rods, and frequently present a granular appearance toward one end or throughout the entire length. The large swellings in the filaments always stain irregularly; usually they are vacuolate and frequently show a distinct tendency to retain the Gram's stain (see plate 1, *11 12, 13*).

The strains which show the swollen filaments on the acid media also show a considerable number of independent bodies similar in structure and size to the swollen portion of the filaments. Their usual oval form gives them a somewhat yeast-like appearance though they are frequently quite irregular and occasionally show a tendency to branch (see plate 1, *9, 10, 11*). Like the swellings in the filaments these bodies usually stain more deeply than the normal rods and usually tend to retain Gram's stain. They always, however, show a considerable irregularity in staining due to vacuoles and the granules. At the same time a few of these yeast-like bodies remain unstained or only very faintly colored.

When these strains are grown upon alkaline media, from pH 8.0 to the maximum alkalinity for growth, equally striking morphological types appear and a larger percentage of the normal rods are, in most strains, replaced by pleomorphic types. The alkaline types closely resemble the acid types of the same strain though the two are generally distinguishable. The filamentous forms on the alkaline media are in some strains long and slender (see plate 1, *7*) but usually they are thicker than the corresponding acid filaments, much more curved or wavey and usually more irregular in staining (see plate 1, *4, 5, 6*). Swollen filaments and irregular swollen yeast-like forms occur in a few strains (see plate 1, *8*) but less frequently than on the acid media.

A striking feature of all these involution forms, both upon acid and alkaline media, is that they are at their maximum in young cultures. It was generally observed that involutions appear after a few hours incubation and are at a maximum in twenty-

four hour old cultures; older cultures have seldom been found to show any greater degree of variability.

It is apparent that these results must depend to a considerable extent upon the stability of the reaction of the media. Rivers (1920) has shown that some strains of *H. influenzae* slightly increase the acidity, other strains slightly increase the alkalinity of blood-broth and Stillman and Bourn (1920) have shown that in blood-broth containing glucose a final pH value of 6.4 is reached by cultures of this organism. The media used in these experiments contain no sugars and are heavily buffered with phosphates and with blood. Potentiometer readings with a hydrogen electrode at various stages in the development of the cultures, moreover, show too slight a change in the acidity during the period of observation to effect the results.

3. Attempts were made to increase the percentage of involutions and the degree of variability from the type forms by continued cultivation upon a medium of an acidity favouring variability. Strain 4, for example, was grown for twenty cultural generations of twenty-four hours each, on blood agar media of three acidities pH 6.0, pH 7.4 and pH 8.5. At the end of this series of generations the cultures on pH 7.4 media were typical small rods as at the start; the cultures on the pH 6.0 media consisted of the same involution types and about the same percentage distribution as at the end of the first twenty-four hour generation on this acidity; and likewise the cultures on the pH 8.5 media presented the same appearances as at the end of the first cultural generation on this media. Other strains gave similar results.

Transfers were made from these cultures which had been for twenty generations on the acid and on the alkaline media, to the standard medium. After a short period of incubation in their first cultural generation the organisms could not be distinguished from those long cultured on the standard medium.

It would appear, therefore, that these morphological variations are produced in direct reaction to the environment and that their appearance is neither appreciably influenced by the previous history of the organism nor do the new forms show any heritable tendency.

TABLE 1

Morphological types of H. influenzae when grown on blood-agar of various hydrogen-ion concentrations

STRAIN	SOURCE	BLOOD-AGAR BUFFERED TO		
		pH 5.5 to 6.0	pH 6.8 to 7.8	pH 8.5 to 8.8
2	Lung	Rods and filaments	All rods	Rods and filaments
5	Nasopharynx	Mostly rods, few filaments, very few yeast-like	All rods	All filaments
6	Nasopharynx	Rods and filaments, very few yeast-like forms	All rods	All filaments
9	Spinal fluid	Mostly rods, few filaments	All rods	Mostly filaments
a	Spinal fluid	Mostly filaments, few yeast-like	All rods	All filaments
b	Spinal fluid	Mostly filaments, few yeast-like	All rods	All filaments
c	Spinal fluid	Mostly filaments	All rods	All filaments
I	Sputum	Mostly filaments	Mostly rods, few filaments	Mostly filaments
J	Sputum	Mostly filaments	Mostly rods, few filaments	Mostly filaments
4	Nasopharynx	Mostly filaments, many yeast-like forms, few rods	Mostly rods, few filaments	Mostly filaments
8	Spinal fluid	Rods and filaments	Mostly rods, few filaments	Mostly filaments
11	Nasopharynx	Rods, filaments, few yeast-like forms	Mostly rods, few filaments	Rods and filaments
Neg.	Sputum	Mostly rods	Mostly rods, few filaments	Rods and filaments
3	Lung	Rods and filaments, few yeast-like	Rods and filaments	All filaments
10	Spinal fluid	Mostly irregular filaments and few yeast-like forms	Rods and filaments	Mostly irregular filaments and few yeast-like forms
7	Spinal fluid	Mostly filaments, few yeast-like forms	Mostly filaments	All filaments

SUMMARY

1. It has been shown that *H. influenzae* when grown upon a standardized blood-agar medium is usually a short rod though many strains show some coccoid or short filamentous forms. Continued cultivation on this standard medium has resulted in very little morphological variation from the initial condition.

2. Extreme pleomorphic forms have been shown to occur in young cultures in media made acid or alkaline close to the growth limiting reaction for the species.

3. The pleomorphic forms occur to the maximum degree after a few hours cultivation on the media promoting their formation and as suddenly disappear when they are transferred to media promoting normal morphology.

The bearing of these results and others of a similar nature upon the life history of bacteria as discussed by Löhnis and Smith, Hort and others, and more significantly, upon the physical chemistry of the protoplasm, will form the subject of subsequent papers.

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EXPLANATION OF PLATE

PLATE 1

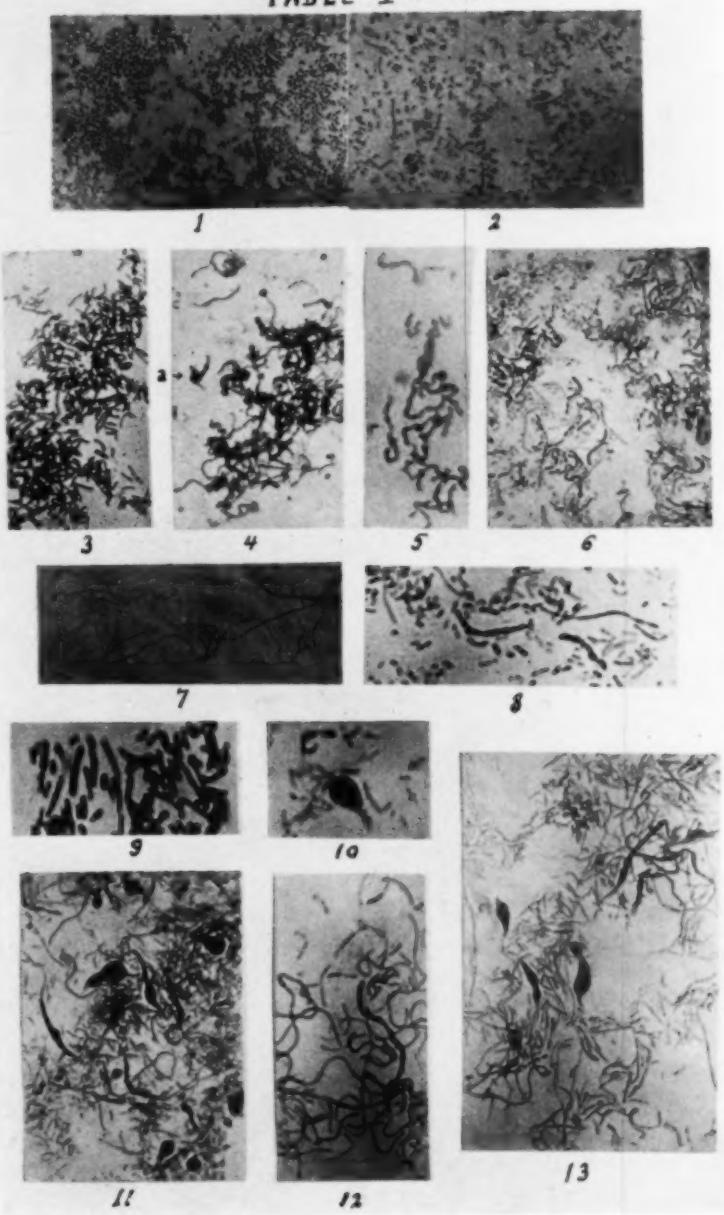
Photomicrographs of smears of *H. influenzae* from cultures on various media, as indicated. The smears in each case were stained by Gram's method counterstained with watery fuchsin. The magnification in all the figures is the same, 1000 diameters.

1–3. Photomicrographs of smears of different strains of *H. influenzae* grown on blood-agar pH 7.6, the standard blood-agar described in the text. *1*, a strain of small rods; *2*, a strain of small rods with a few short filaments; *3*, a strain of short filaments with a few small rods.

4–8. Photomicrographs of smears of different strains of *H. influenzae* grown on strongly alkaline blood-agar. *4*, short tapering filaments with a few swollen types, some showing a tendency to branch, as at *a*; *5*, long much twisted filaments. *6*, short irregularly stained filaments; *7*, very long slender filaments; *8*, irregular filaments and rods.

9–13. Photomicrographs of different strains of *H. influenzae* grown upon strongly acid blood-agar. *9*, Swollen rods and filaments and a branching yeast-like body; *10*, a large yeast-like body showing a bar-like granulation; *11* and *13*, rods and long filaments, many much swollen at the ends or in a central position; *12*, irregular rods and long filaments.

TABLE I



(Reed and Orr. H. influenzae)

VARIATIONS IN HYDROGEN SULPHIDE PRODUCTION BY BACTERIA

F. W. TILLEY

From the Biochemic Division, U. S. Department of Agriculture, Bureau of Animal Industry

Received for publication June 1, 1922

In a paper already published (1921) the writer has called attention to the effect of peptone on indol production. In the present paper there are reported experiments dealing with the effect of peptone on the production of hydrogen sulphide by bacteria.

EXPERIMENTAL WORK

The culture medium employed in nearly all of the experiments was lead acetate agar prepared according to the directions given by Jordan and Victorson (1917), with some modifications, as follows: 3 per cent of peptone was dissolved by boiling in fresh meat infusion; after filtration 1.5 per cent agar was added and dissolved by heating in the autoclave, the reaction was adjusted to pH 7.2 and the medium tubed and sterilized in the autoclave. After sterilization the tubes were cooled to 43°C. and 3 drops of a freshly made 10 per cent solution of lead acetate added to each tube. The tubes were then shaken vigorously and rapidly cooled. The cultures used were grown in peptonized beef broth and the lead acetate tubes were each inoculated with one loopful of broth culture, the loop being introduced between the agar and the wall of the tube and passed down to the bottom. It was found that the best results were obtained when young broth cultures were used for inoculating the agar; twenty-four hour cultures were usually employed, but forty-eight-hour cultures gave results just about as good and were used when more convenient.

The results of a number of such experiments are given below in table 1. Numbers are used to indicate the different varieties of peptone while letters with the numbers indicate different samples of the same varieties of peptone.

Table 1 shows that there are decided differences between the different peptones and between different samples of the same

TABLE 1

Comparative hydrogen sulphide formation with different peptones

Experiment 1

CULTURES	PEPTONES				
	1A	2	3	4A	5
Proteus 4	−	+++	−	−	++
Proteus 19	−	+++	−	−	++
Bact. suipestifer 360	−	−	−	−−	−
Bact. suipestifer 416	−	+++	−	+++	++
Bact. typhosum A	−	+++	−	+++	+++
Bact. typhosum D	−	−	−	−	−

Experiment 2

CULTURES	PEPTONES				
	1B	1C	1D	4B	6
Proteus 4	+	+	++	+++	+++
Proteus 19	+	+	++	+++	+++
Bact. suipestifer 360	−	−	−	−	−
Bact. suipestifer 416	+	−	++	+++	+++
Bact. typhosum A	+	+	++	+++	+++
Bact. typhosum D	−	−	−	−	−

− = Negative reaction. + = Weak reaction. ++ = Good reaction. +++ = Strong reaction.

peptone. It also shows that different strains of the same organism may produce different results.

The differing results with different strains of the same organism seemed to warrant more study and as a result there was discovered a curious correlation between hydrogen sulphide production in lead acetate agar and growth in a synthetic medium having the following composition:

Sodium ammonium hydrogen phosphate.....0.2 per cent
Glucose.....1 per cent
Brom cresol purple.....5 drops of 0.04 per cent solution per tube

The reaction was not adjusted. The medium was sterilized in ordinary bacteriological tubes at 15 pounds pressure for fifteen to twenty minutes. Growth caused acid production and a change in color from purple to yellow.

TABLE 2

Hydrogen sulphide production by different strains of Bact. typhosum and Bact. suipestifer

ORGANISM	H_2S REACTION	SYNTHETIC MEDIUM
Bact. suipestifer		
Number 320	Positive	Growth
Number 334	Negative	No growth
Number 346	Negative	No growth
Number 360	Negative	No growth
Number 407	Positive	Growth
Number 416	Positive	Growth
Number 420	Positive	Growth
Number 486	Negative	No growth
Bact. typhosum		
A	Positive	No growth
HL	Positive	No growth
C	Positive	No growth
D	Negative	Growth
K 110	Negative	Growth
K 111	Positive	No growth
K 114	Positive	No growth

The correlation above mentioned is shown in table 2, which gives the consolidated results of a number of experiments. The peptone used in the lead acetate agar was peptone 2; the results under synthetic medium are those obtained after twenty-four hours' incubation; the results under H_2S reaction represent observations made after one to three days' incubation.

The correlation shown above between the ability of bacteria to produce H_2S and their ability to grow in the synthetic medium appears to be an interesting coincidence rather than evidence of any fundamental connection between the two phases of bacterial

metabolism concerned. As the table shows, the relation between H_2S formation in lead acetate agar and ability to grow in the synthetic medium is exactly opposite in the case of *Bact. typhosum* to what it is in the case of *Bact. suipestifer*. In addition it may be stated that three strains of *Bact. coli* all failed to produce H_2S and all grew in the synthetic medium three strains of *Bact. dysenteriae* all failed to produce H_2S and two failed to grow in the medium while the third grew very slowly, four strains of *Bact. enteritidis* all produced H_2S and all grew in the synthetic medium, eight strains of Proteus all produced H_2S and all grew in the synthetic medium, although three were slow in developing, and 15 strains of *Bact. paratyphosum* B all produced H_2S and all grew promptly in the synthetic medium.

It will thus be seen that although ability to produce H_2S and ability to grow in the synthetic medium are not related in a uniform way in the different species of organisms mentioned above, the relation between them is quite constant so far as any one species is concerned.

This is by no means an invariable rule, since exceptions to it are frequently observed. But as far as *Bact. suipestifer* is concerned, out of more than 150 strains recently examined in this division, only 6 failed to conform to the rule that strains which produce H_2S also grow in the synthetic medium while those which do not produce H_2S do not grow in the synthetic medium.

In regard to the relation between hydrogen sulphide production by bacteria and the chemical composition of the various peptones it may be stated that the writer has attempted in connection with the work herein recorded to follow the example of his previous work upon indol production, where everything depended upon the presence or absence of tryptophan, and show that the relative value of peptones for hydrogen sulphide production depended upon the amount of cystin present. It was soon found, however, that the problem was not as simple as it seemed and it was therefore made the subject of a new line of inquiry, the results of which the writer hopes to present before long in another paper.

It can be stated at the present time, however, that experiment has shown that the addition of cystin to lead acetate agar made

with peptone 1 causes the production of H_2S by *Bact. coli* and other organisms which are usually lead negative; while on the other hand the addition of a small amount of sodium thiosulphate makes it possible to obtain results similar in every way to those obtained with peptone 2.

DISCUSSION

The fact that different peptones will yield different amounts of hydrogen sulphide has previously been mentioned by Myers (1920) and by Thompson (1920–21). Each of them used Difco, Fairchild and Witte peptones, which are also included among the 6 used by the present writer. Myers used fluid media while Thompson used a lead acetate agar prepared by a method differing somewhat from that used by the writer. Neither of them has reported any difference between different strains of the same organism so far as H_2S production is concerned. In the case of *Bact. suipestifer* it seems likely that these differences between strains are the cause of conflicting statements in the literature. For example, according to Jordan and Victorson (1917) *Bact. suipestifer* does not produce hydrogen sulphide, while according to Buchanan (1916) and McFarland (1919) it does produce hydrogen sulphide.

The writer has designated the various peptones by numbers and letters instead of giving names, because his object was not to show the superiority or inferiority of any particular varieties of peptones but rather to show what a variable quantity "peptone" really is,—so variable, indeed, that if the names of the peptones were given the information would be more likely to mislead the reader than to help him.

As evidence of this it may be noted that Myers and Thompson ascribe different relative merits to the 3 peptones used by them, while the present writer's work would indicate still different relative values. What else could be expected if we stop to think that, after all, "peptone" in the bacteriological sense of the word is only a name for an indefinite mixture of proteoses, peptones, polypeptides and various other compounds varying enormously

in composition, depending on the materials used and the method of manufacture?

It would seem that until such time as we possess more exact knowledge of the composition of our culture materials, correct results will be possible only if all new culture materials are tested before use and found to be suitable for the particular work to be done.

CONCLUSIONS

1. When used in lead acetate agar different peptones now available in this country yielded extremely variable results so far as hydrogen sulphide production is concerned. In some instances different samples of the same peptone differed from each other.

2. The different strains of *Bact. suipestifer* and *Bact. typhosum* were found to be divided into two groups, those in one group producing hydrogen sulphide while those in the other group failed to produce hydrogen sulphide.

3. On account of variation in the composition of commercial peptones and variation in the hydrogen sulphide producing power of different strains of the same organism, it is advisable before using new culture materials in work with unknown organisms to test these culture materials with known strains of known organisms.

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A COMPARISON OF THE ZIEHL-NEELSEN AND SCHULTE-TIGGES METHODS OF STAINING TUBERCLE BACILLI

HYMAN L. SHOUB

New York City

Received for publication August 2, 1922

I have always been impressed with the fact that many patients who exhibit signs of pulmonary tuberculosis clinically and by x-ray plates give a negative test for tubercle bacilli constantly or most of the time.[1] There are, of course, various factors which are beyond control that cause this condition. However, there seemed to be one factor, the staining method used, which might be responsible for some of it. I therefore began to search the literature and found that other workers in this field have been similarly impressed, and have accused the Ziehl-Neelsen method of not always staining the tubercle bacilli; or stating it in another way, that tubercle bacilli are not all acid fast.

In my search I have encountered various modifications of the Ziehl-Neelsen method, and some other methods of staining tubercle bacilli, and the following have been tried:

1. The Hermann method, which is a crystal violet stain.[2]
2. The Cronberger method, which is a modification of the Ziehl-Neelsen method.[3]
3. The Schulte-Tigges method,[4] to be described later.

[1] The work described in this article was done at the United States Public Health Service Hospital no. 32, Washington, D. C., during the months of March, April, May and June, 1921. The method described has been in use at this Hospital ever since.

[2] A comparison of the Ziehl-Neelsen and Hermann methods of staining tubercle bacilli, Abst. Bact., **3**, no. 1–4.

[3] Cronberger method, Zeitschrift for T.B., 1916, **25**, no. 2, p. 109.

[4] Schulte-Tigges, H. Deutsche Mediz. Wochensch., October 28, 1920, **46**, no. **44**, p. 1225.

All the above methods have been tested out against the Ziehl-Neelsen method with known positive, negative, and doubtful sputums. In the preliminary tests, it was found that the Schulte-Tigges method gave more encouraging results and it was therefore selected for further study.

The procedure for testing a sputum was as follows: The sputum was well mixed with a glass stirring rod; a portion of it was rubbed between two slides to insure equal distribution of the specimen on both slides; they were dried and then fixed by heat. One of them was stained by the Ziehl-Neelsen method, and the other by the Schulte-Tigges method. The smear should be fairly heavy.

The procedure for staining was as follows:

Ziehl-Neelsen method

1. Flood the slide with carbol-fuchsin (10 cc. of saturated solution of basic fuchsin in 95 per cent alcohol, and 90 cc. of 5 per cent carbolic acid).
2. Heat the slide to steaming and continue the heating for five minutes.
3. Wash the excess stain with tap water, and decolorize in acid alcohol (1 cc. of concentrated HCl in 99 cc. of 70 per cent alcohol).
4. Wash with tap water and counterstain with Methylene blue (saturated aqueous solution of methylene blue).
5. Wash with tap water, dry and examine.

Schulte-Tigges method

1. Flood the slide with carbol-fuchsin (10 cc. of saturated solution of basic fuchsin in 95 per cent alcohol, and 90 cc. 5 per cent carbolic acid).
2. Heat the slide to steaming and continue the heating for *one* minute. Avoid excessive heating.
3. Wash off the excess stain with tap water, and decolorize in sodium sulphite (10 per cent aqueous solution of sodium sulphite). This solution should be renewed at least once a week and if convenient twice a week.
4. Wash *thoroughly* with tap water, and counterstain with picric acid (saturated aqueous solution of picric acid).
5. Wash with tap water, dry, and examine. The tubercle bacilli are red, the background is pinkish yellow.

It should be mentioned here that in the original description of the method the excess picric acid is not washed off; however I found that better results are obtained by washing after counterstaining.

It will be noted that the new method is not more difficult than the Ziehl-Neelsen method.

All the slides when shown to be positive for tubercle bacilli, were rated by the Gaffky method; and a record was made of the average number of organisms per field on an average of ten fields. The Gaffky method of recording the results of positive sputum is as follows:

Gaffky's scale

	The values used in this test
I. Only one to four bacilli in whole preparation	0.2
II. Only one on an average in many fields	0.5
III. Only one on an average in each field	1.0
IV. Two to three on an average in each field	2.5
V. Four to six on an average in each field	5.0
VI. Seven to twelve on an average in each field	9.5
VII. Thirteen to twenty-five on an average in each field	19.0
VIII. About fifty on an average in each field	50.0
IX. About a hundred on an average in each field	100.0

During the four months in which this method was being tried out, we examined about 800 sputums, 244 of which were positive with the new stain and only 183 positive with the Ziehl-Neelsen stain, or for every 100 positives by the new method only 75 were positive by the Ziehl-Neelsen. Another important factor is the higher Gaffky reading with the new stain which means more organisms per field. In all this investigation no sputum was even found positive by the Ziehl-Neelsen stain and negative with the new stain, while the reverse was true in 33 per cent of the tests.

Figuring as in table 1, it was found that the total number of organisms of the specimens examined was 657 by the Ziehl-Neelsen method, and 3658 by the Schulte-Tigges method.

Therefore the relation between the two stains is $\frac{3658}{657} = 5.57$, or the new method is more than five times as good.

TABLE 1

Showing the results of the tests with both stains, on specimens from patients 3 and 9

PATIENTS NUMBER	DATE OF TEST	NUMBER OF ORGANISMS PER FIELD	
		Ziehl-Neelsen	Schulte Tigges
3	May 25, 1921	2.5	5.0
	June 7, 1921	9.5	100.0
	June 13, 1921	0.0*	1.0
	June 23, 1921	5.0	19.0
	June 24, 1921	1.0	9.5
	June 25, 1921	0.5	9.5
	June 27, 1921	0.2	5.0
	July 11, 1921	2.5	19.0
	July 12, 1921	0.0*	5.0
	July 13, 1921	0.5	19.0
Total		21.7	192.0
	$\frac{19.20}{2.17} = 8.85$		
9	June 3, 1921	0.2	2.5
	June 13, 1921	0.0*	0.5
	June 23, 1921	0.5	19.0
	June 24, 1921	0.0*	5.0
	June 25, 1921	0.2	1.0
	June 29, 1921	0.5	1.0
	July 1, 1921	0.0*	2.5
	July 7, 1921	0.5	19.0
	July 9, 1921	0.5	9.5
	July 9, 1921	0.5	19.0
	July 11, 1921	0.5	9.0
	July 12, 1921	0.0*	0.5
	July 13, 1921	0.0*	50.0
	July 14, 1921	2.5	50.0
	July 21, 1921	0.0*	0.2
	July 22, 1921	0.5	2.5
	July 25, 1921	0.5	50.0
Total		16.9	241.2
	$\frac{241.2}{6.9} = 34.9$		

* Note that this is a negative.

THEORETICAL

It is rather speculative to give any reason for the above results. However, the following points may be discussed here:

1. The acid fastness of the tubercle bacilli

Some people share the opinion that the tubercle bacilli are not all acid fast. Our present work does not seem to corroborate this opinion, and if it does, it is only very mildly. This may be seen from the following table.

TABLE 2

Showing all the negatives (60) with the Ziehl-Neelsen and the corresponding Gaffky's by the Schulte-Tigges method

	AVERAGE NUMBER OF ORGANISMS PER FIELD							
Patient number	Z. N.	S. T.	Patient number	Z. N.	S. T.	Patient number	Z. N.	S. T.
1	0.0*	2.5						
				0.0*	0.5		0.0*	0.2
3	0.0*	1.0		0.0*	50.0		0.0*	0.5
	0.0*	5.0	10	0.0*	2.5		0.0*	9.5
				0.0*	19.0		0.0*	9.5
4	0.0*	1.0		0.0*	5.0	17	0.0*	50.0
							0.0*	1.0
5	0.0*	0.5		0.0*	0.5		0.0*	5.0
				0.0*	1.0		0.0*	2.5
	0.0*	0.2		0.0*	2.5		0.0*	9.5
	0.0*	1.0		0.0*	0.2			
7	0.0*	0.2	12	0.0*	0.2		0.0*	9.5
	0.0*	0.2		0.0*	9.5	18	0.0*	50.0
	0.0*	0.5		0.0*	1.0		0.0*	1.0
	0.0*	0.2		0.0*	0.2			
						19	0.0*	2.5
	0.0*	1.0		0.0*	9.5			
8	0.0*	0.5		0.0*	2.5		0.0*	2.5
	0.0*	19.0	13	0.0*	5.0		0.0*	0.2
				0.0*	0.5	20	0.0*	0.5
	0.0*	0.5					0.0*	19.0
	0.0*	5.0	16	0.0*	0.2		0.0*	0.2
9	0.0*	2.5						
	0.0*	0.5	17	0.0*	5.0	21	0.0*	0.5
	0.0*	50.0						
	0.0*	0.2		0.0*	19.0		0.0*	9.5
Total.....		91.5			133.8			183.1

$$\text{Grand total} = 408.4$$

$$\frac{408.4}{60 \text{ (number of negatives)}} = 6.60$$

In examining the results of table 2, we find that the new method gives, on an average, 6.60 organisms per field, while the Ziehl-Neelsen method gives none per field. However, 6.60 is not far from our figure above (5.57), and it would therefore appear that the negatives are not to be accounted for by lack of acid fastness. It should further be mentioned that 75 per cent of the results fall within the range of 5 and less than 5 organisms per field.

2. The counterstain

In several cases, the Ziehl-Neelsen method was tried by using picric acid as a counterstain, and it was found that in every case it gave somewhat higher results than with the methylene blue counterstain. The counterstain is therefore a responsible factor to some degree.

3. The decolorizer

It seems that the sodium sulphite in decolorizing the slide also clarifies it, and therefore exposes more organisms.

Besides the advantges of this stain in exposing more positives and more organisms per field, it is an advantage to dispose of the use of the alcohol as a decolorizer.

CONCLUSION

1. The Schulte-Tigges method of staining tubercle bacilli is not more difficult than the Ziehl-Neelsen method; it is easier.
2. It gives about 33 per cent more positives.
3. It exposes over five times as many organisms.
4. It does away with the use of alcohol.

PHYSIOLOGICAL YOUTH IN BACTERIA[1]

JAMES M. SHERMAN AND WILLIAM R. ALBUS

From the Research Laboratories of the Dairy Division, United States Department of Agriculture, Washington, D. C.

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INTRODUCTION

One of the outstanding features in the development of the biological sciences during recent years has been the conception of the unity of living matter: the recognition of the biological, chemical and physical laws which govern all forms of life.

However, in considering some of the problems of life it is frequently customary to think in quite different terms when viewing these phenomena among unicellular organisms than when the metazoa are considered. For example, the period of youth, as it is known among higher animals, is not ordinarily considered to have a physiological counterpart in those unicellular organisms which reproduce, so far as is known, only by cell division. Probably a fair statement of the current view is that of Mitchell (1912) who says, after describing the process or reproduction by cell division in amoebae: "The two amoebae may be called young animals in the sense that they have just come into existence as new individuals, but nothing in their tissues or characters distinguishes them from their parent. So far as the period of youth has any interest or significance, these animals escape it."

It would seem, a priori, that the newly formed bacterial cells should show some physiological characteristics which would differentiate them from more mature cells. It would not, perhaps, be carrying the comparison too far to enquire as to whether the young of an unicellular organism, like the young

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of higher organisms, are more sensitive to the influence of the environment than are the more mature cells of the same species. However, experimental evidence of such a nature, so far as we are aware, is entirely wanting. In fact, the observation of Chick (1908) that young cultures of the paratyphoid organism, under the conditions of her experiments, are more resistant to phenol than old cultures has been interpreted by some as indicating a greater general vigor and resistance in young bacterial cells. We do not think the fact that young cells may not enter so readily into chemical combination with a disinfectant justifies the view that they are more resistant to the physical hazards of life; and it certainly does not seem logical to infer that the young cells are born, so to speak, more perfectly adjusted to the environment than are their elders.

Our knowledge concerning the properties of bacteria has been accumulated mainly through the study of what might be termed mature cultures; that is, cultures twenty-four hours or more of age. As is well known, at this age cultures of most of the best known bacteria have passed their period of most rapid growth and have settled down to a more or less inactive state so far as reproduction is concerned. Whether in this state of reproductive latency there is practically no multiplication of cells, or whether the reproduction and mortality are about balanced, there can be no doubt that the rate of cell division is extremely slow as compared with that of a culture during its period of logarithmic growth, and that the cells of the older culture are relatively of much greater age than are those occurring during the period of maximum rate of multiplication. We have, therefore, attacked this problem by making comparative studies of old cultures, and of young cultures taken during their period of most rapid growth.

EXPERIMENTAL

It is well known that brief exposure to cold above the freezing point of the medium in which the bacteria are suspended causes little or no reduction in the number of bacteria subjected to such treatment. This is verified by the data given in table 1

from tests in which cultures of *Bact. coli* six and eleven days old, grown in 1 per cent pepton at laboratory temperature, were diluted in distilled water at a temperature of 2°C. Plates were made from these dilutions immediately and then again after an one hour exposure at this temperature. It will be seen from the data given in the upper half of table 1 that in neither experiment was there a reduction in the number of bacteria.

For plating in this experiment, as in the other experiments to be reported, the medium consisted of the standard extract pepton agar and the plates were incubated at 33°C. for three days.

TABLE 1

Effects of exposure in distilled water at 2°C. upon mature and young cells of Bact. coli

EXPERIMENT NUMBER	AGE OF CULTURE	TEMPERATURE INCUBATED	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
			At beginning	After one hour
1	11 days	Laboratory	607	606
2	6 days	Laboratory	720	710
1	4 hours	37°C.	530	155
2	4½ hours	37°C.	373	247

Turning now to the effect of cold upon young bacterial cells, it will be seen from the data contained in the lower half of table 1, in which actively growing cultures of *Bact. coli* (in 1 per cent pepton at 37°C.) were treated in a similar manner, that there was a distinct mortality among the young bacterial cells when subjected to a sudden cooling.

From the results given in table 1 alone it would not be safe to draw the conclusion that chilling by itself was responsible for the death of the actively growing cells, since we measured, in those cases, the mortality in distilled water dilutions held at low temperature. The experiments were conducted in that manner instead of cooling the original cultures in order to eliminate any possible errors in sampling or in making the necessary dilutions for the counts. It is recognized that a part of the mortality might have been due to the sudden change from a nutrient

medium to distilled water; this, however, does not vitiate in the least the indicated fact that there exist physiological differences between young and mature bacterial cells.

In order to test the effect of cold uncomplicated by such an additional factor, another experiment was conducted in which similar old and young cultures were diluted in sterile 1 per cent pepton instead of sterile distilled water. In this test the dilutions of the cultures in pepton solution were held for three hours at 2°C.; counts were made at the beginning and after the first, second and third hours. As the platings at the different periods were all made from the same broth dilutions, the possibilities of error through sampling and subsequent dilutions were again eliminated; and since the dilutions were made in broth of the same composition as that in which the cultures were grown,

TABLE 2

Effect of exposure at 2°C. upon mature and young cells of Bact. coli

AGE OF CULTURE	TEMPERATURE INCUBATED	NUMBER OF BACTERIA PER CUBIC CENTIMETER OF BROTH DILUTION			
		At beginning	After one hour	After two hours	After three hours
12 days	Laboratory	970	930	1,010	970
4 hours	37°C.	3,420	1,460	1,160	980

there would seem to be in this case no probable detrimental factor concerned other than that of temperature. As is seen from table 2, there was again found to be a marked mortality among the cells of the actively growing culture, while in the old culture there was no measurable decrease.

In an effort to find another treatment of a mild nature which would cause a more marked destruction among young than among older bacterial cells, exposures in dilute solutions of NaCl were tried. Winslow and Falk (1919) have shown that the mortality of *Bact. coli* in water is distinctly increased by the addition of 4.5 per cent NaCl. This, in view of the preceding experiments upon the effect of low temperature, encouraged us to believe that a lower concentration of NaCl might be found which would be distinctly lethal for young cells, while having little effect upon

mature cultures. By using a 2 per cent solution of NaCl in distilled water as a dilution medium it was found that mature cells showed little if any mortality from an one hour exposure at laboratory temperature. In the first four lines of table 3 are given the results of such experiments conducted with cultures which had been incubated twenty-one hours at 37°C., two days at laboratory temperature, and seven days at laboratory temperature; the data obtained with these cultures show little if any effect of such treatment.

Contrary to the results obtained with mature cultures, the data given in the last three lines of table 3 show a very marked

TABLE 3

Effect of exposure in 2 per cent NaCl upon mature and young cells of Bact. coli

EXPERIMENT NUMBER	AGE OF CULTURE	TEMPERATURE INCUBATED	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
			At beginning	After one hour
1	21 hours	37°C.	890	770
2	2 days	Laboratory	378	356
3	7 days	Laboratory	1,470	1,410
4	7 days	Laboratory	880	940
1	3½ hours	37°C.	2,530	27
2	3½ hours	37°C.	1,110	72
3	3½ hours	37°C.	2,190	55

mortality among young cells of *Bact. coli* when exposed for one hour at laboratory temperature in a 2 per cent solution of NaCl.

As a further confirmation of the foregoing evidence that newly formed bacterial cells are more delicate than older ones, the effect of a short exposure to heat at a relatively low temperature upon old and young cultures was tried. In table 4 are given results obtained by heating *Bact. coli* in 1 per cent pepton (pH 7.0) to 53°C. for twenty minutes. As is well known, there is a rapid destruction of bacteria at all temperatures above their limit of growth, but an examination of these data shows a much greater mortality among the young than among the mature cells or *Bact. coli*.

The experiments thus far reported were conducted entirely with *Bact. coli* and it is of course recognized that it would not be safe to draw too general conclusions from the work on this organism alone; we have, therefore, made similar tests with a stock culture of *Proteus*. This culture is one of those which was extensively studied by Rogers (1921) and belongs to the type that produces carbon dioxide without hydrogen in its fermentation of glucose. It also ferments maltose and thus should probably be designated as *Proteus vulgaris* in accordance with the nomenclature of Wenner and Rettger (1919). In table 5 are given the results obtained from exposures of young and mature cultures of *Proteus* in 2 per cent NaCl solutions for one hour at laboratory temperature. In this case there was no

TABLE 4

Effect of exposure at 53°C. for twenty minutes upon mature and young cells of Bact. coli

AGE OF CULTURE	TEMPERATURE INCUBATED	NUMBER OF BACTERIA PER CUBIC CENTIMETER		RATIO BEFORE : AFTER
		Before heating	After heating	
7 days	Laboratory	1,470,000,000	97,000,000	15:1
3½ hours	37°C.	27,800,000	24,600	1,130:1

decrease in the number of cells from the older culture whereas the young cells showed a definite mortality. Although the contrast in this case was not so marked as in the experiments reported above with *Bact. coli*, it is probable that by increasing the salt concentration, or lengthening the time of exposure, just as striking results could be obtained with *Proteus vulgaris*.

An experiment made upon the effect of mild heating upon *Proteus vulgaris*, which is reported in table 6, gave data confirming those obtained in the studies with *Bact. coli*. In these tests cultures three and one-half and twenty-six hours old at 37°C. (in 1 per cent pepton, pH 7.0) were heated to 50°C. for ten minutes. An examination of the results of plate counts made before and after this treatment shows again a greater mortality in the young than in the mature culture.

Since there appears to be a difference between old and young bacterial cells with respect to their ability to withstand certain deleterious physical agents, it would seem hopeful to attempt to find some differences in the properties of their protoplasms which could be detected by some convenient test. The isoelectric point suggested itself as a possibility, so young and old cultures were tested for their agglutinability with acid. This worked well since the young cells proved to be very resistant to agglutination while the mature cells were readily flocculated when the

TABLE 5

Effect of exposure in 2 per cent NaCl upon mature and young cells of Proteus vulgaris

AGE OF CULTURE	TEMPERATURE INCUBATED	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
		At beginning	After one hour
hours	°C.		
26	37	391	396
3½	37	1,120	570

TABLE 6

Effect of exposures at 50°C. for ten minutes upon mature and young cells of Proteus vulgaris

AGE OF CULTURE	TEMPERATURE INCUBATED	NUMBER OF BACTERIA PER CUBIC CENTIMETER		RATIO BEFORE : AFTER
		Before heating	After heating	
hours	°C.			
26	37	440,000,000	183,000,000	3:1
3½	37	10,100,000	116,000	87:1

hydrogen ion concentration of the medium was increased to a pH value of about 3.8.

The results of a typical experiment are given in table 7. In this test the cultures were grown in 1 per cent pepton-0.20 M NaCl broth; the old culture was diluted sufficiently with sterile broth to give it about the same turbidity as the young culture; and the agglutination was effected by the addition of N/14 H_2SO_4 to the desired pH value. Readings were made after one and one-half hours at laboratory temperature.

Similar results have been obtained with cultures grown in broths of other compositions as well as with cells taken from agar slopes and suspended in 1 per cent NaCl solution.

The fact that a difference exists in the acid agglutinability of young and mature bacterial cells is, we think, significant. However, the demonstration of this point is not new since MacGregor (1910), studying serum agglutination, found that meningococci grown for more than forty-eight hours became so easily agglutinable as to flocculate with normal serum; while Gillespie (1914), working on the acid agglutination of pneumococci, found young cultures to be much more resistant to agglutination than were older ones.

We are aware of the fact that *Bact. coli* has been credited by some investigators as being non-agglutinable with acid; concerning this point, no comment is called for in the present case other than to state that the culture employed was of the typical colon type with respect to its gas ratio (Rogers, Clark and Davis, 1914), the methyl red test (Clark and Lubs, 1915), and the Voges-Proskauer reaction (Levine, 1916).

TABLE 7

Acid agglutination of Bact. coli

AGE OF CULTURE	AGGLUTINATION AT pH VALUE OF				
	6.0	4.1	3.8	3.4	3.0
hours					
4	–	–	–	–	–
24	–	+	+++	+++	++

While it was not considered that the points established in this work are irreconcilable with those of Chick (1908) upon the destruction of *Bact. paratyphosum* by phenol, it seemed desirable to touch on that point in our work. Table 8 gives the data obtained in one experiment in which the rate of destruction of four and twenty-four hour cultures of *Bact. coli* in 0.5 per cent phenol was tested. In this experiment the cultures were grown in 1 per cent pepton and the phenol added to the pepton culture. The twenty-four hour culture was diluted about one hundred

times with sterile 1 per cent pepton, before the phenol was added, in order to have approximately the same number of cells present in each case and also to have, so far as possible, the same conditions with respect to reaction and metabolic products.

These results indicate that, in the case of the organism studied, the young cells are more sensitive to phenol than are the mature ones. A repetition of the experiment, using the same technique, gave similar results.

In reviewing the work of Miss Chick it is noted that she did not find the young cultures of the paratyphoid organism more resistant to phenol when tested after three hours incubation on

TABLE 8

Action of 0.5 per cent phenol upon young and mature cells of Bact. coli

TEST NUMBER	AGE OF CULTURE	NUMBER OF BACTERIA PER CUBIC CENTIMETER	
		Before treatment	After three minutes in 0.5 per cent phenol
	hours		
1	4	1,240,000	300
	24	4,650,000	42,600
2	4½	2,400,000	430
	24	6,900,000	10,800

the *first* transfer, but when the *third* generation of three hour cultures was tested they appeared to be more resistant. Since the amount of inoculation used in transferring the cultures was not given, and since the growth curve was not followed, it is difficult to interpret her results in terms of our own. However, it is not impossible, in view of the fact that transfers from actively growing cultures give no lag (Barber, 1908), that the cultures in Miss Chick's experiments had completed the period of rapid multiplication. As Chesney (1916) has well said, "it would seem necessary to obtain accurate knowledge of the growth curve of a culture before the terms 'old' or 'young' are applied to it."

DISCUSSION

The demonstration of a definite physiological youth in bacteria is in full accord with modern developments in general biology as well as with the established facts of bacteriology. The work of Child (1915) establishes definitely the fact that the condition of physiological youth and the process of rejuvenescence may be entirely divorced from sexual processes. His demonstration that regenerating parts of a planarian are physiologically younger than the original organism and that old planarians can be rejuvenated by starvation show clearly that youth in a physiological sense is not necessarily the result of a sexual union. In keeping with this also is the work of Woodruff and Erdmann (1914) with non-conjugating paramecia. In their studies it was found that when a culture of paramecium was carried through successive generations without conjugation the organisms were periodically observed to undergo an internal reorganization process during which the cellular contents were morphologically disintegrated and then redifferentiated. This process, which was named by Woodruff and Erdmann endomixis, appears in a sense to rejuvenate the organisms since it is followed by a more rapid multiplication rate, as is the case following conjugation.

It is perhaps going too far to regard endomixis as a rejuvenating process, especially in view of the recent results of Woodruff (1921) with a new species, *Paramecium calkinsi*, which did not undergo endomixis when carried for a year without conjugation, notwithstanding the fact that it exhibited characteristic growth cycles. Whether this is true or not, the work of Child appears to leave no doubt as to the fact that physiological youth can be achieved by other means than sexual processes.

It would now seem that the conception of young tissue in a physiologic sense should be extended to the organisms which reproduce by simple cell division, the newly formed cells being younger than the parent cell from which they were derived. Such a view is indeed already held by Child (1915) as is indicated by the statement that, "it is certain that the new individuals which arise by division or budding from other individuals or

from experimentally isolated pieces are to some extent physiologically younger than the parent individual from which they arose." The present work, we believe, adds experimental weight to such a view. That morphological differences exist between bacterial cells from mature cultures and from cultures during the period of rapid growth has been well established by the careful works of Clark and Ruehl (1919) and Henrici (1921); that physiological differences should exist would seem only logical in view of the known morphological facts.

Recognition of the fact that young bacterial cells may differ considerably in physiological characteristics from cells of mature cultures should lead to a number of applications in bacteriology. We have found, for example, that the errors which are encountered in the plate count may be quite different dependent upon whether the sample under examination is from a mature culture or from one during its period of rapid growth.

Since the young of bacteria, like the young of higher forms of life, are more susceptible to the hazards of their environment, it is not improbable that in the struggle for existence among these organisms there occurs a certain mortality among the young cells—an "infant mortality" so to speak. If such exists, another factor must be considered in discussions of the relative merits of direct and cultural methods of bacterial enumeration.

As it is probable that the observed differences between young and old bacterial cells are in some way associated with differences in permeability and water content it would seem likely that they would differ in their abilities to stimulate antibody production; such a difference might find ready application in the production of active immunity. The applicability of these facts in agglutination phenomena is already known to some extent and probably deserves wider appreciation.

To cite another example, in an entirely different field of bacteriology, the pasteurization of milk may be mentioned. As has been shown by Ayers and Johnson (1913), the efficiency of pasteurization can not be judged, as was formerly thought, on the basis of the percentage of bacteria destroyed. It is found that in the case of milks with high bacterial counts (in which

there has necessarily been an extensive growth of bacteria) it is easy to obtain an efficiency above 99 per cent, while with milks of low bacterial count (in which there has been little or no proliferation of cells) no such percentage destruction is obtained. It is of course recognized that this fact is probably due in the main to the differences in the types of bacteria predominating in these classes of milks, but it is possible that the physiological condition of the cells with respect to age also plays some part.

SUMMARY

The results of experiments are reported which indicate that physiological differences exist between young and mature bacterial cells; that the newly formed cells pass through a period of physiological youth.

Tests with *Bacterium coli* have shown that cells taken from a culture of this organism during its period of rapid growth are sensitive to certain mild exposures which cause no mortality among the cells from an older culture. Treatments which have yielded such results are brief exposures to cold (2°C.), and exposures in a dilute solution of NaCl (2 per cent).

Experiments conducted upon the effect of heating to relatively low temperatures showed that young bacterial cells are more rapidly destroyed than are the older ones. Other tests indicated that the same is true of the action of phenol.

That there actually exists a difference in the physico-chemical condition of bacterial cells of different ages is indicated by differences in their agglutinability, as was revealed by acid agglutination tests.

Repetition of some of these experiments in which young and old cells of *Proteus vulgaris* were used instead of *Bacterium coli* gave similar results with this organism.

The biological significance of these results and some of their possible practical applications are discussed.

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STUDIES ON SALT ACTION

VI. THE STIMULATING AND INHIBITIVE EFFECT OF CERTAIN CATIONS UPON BACTERIAL GROWTH[1]

MARGARET HOTCHKISS

From the Department of Public Health, Yale School of Medicine

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The object of the present study was to conduct a general survey of the effect of various cations upon the growth of bacteria, under somewhat widely varied conditions of concentration. Previous work, except that of Lipman (1909), Brooks (1920), Winslow and Falk (1918, 1918a), Falk (1920), and Holm and Sherman (1921), dealt only with limiting toxicities and ignored possible stimulating effects; and the investigators cited studied only a very few salts. It was felt that it would be of real interest to undertake a more comprehensive survey of the stimulating, as well as the toxic, concentration of the various chemical elements. A series of inorganic compounds was used in which different cations were combined with the same anion. At it is presumed that materials in solution are most likely to be reactive with protoplasm, the chlorides were chosen because they form a large series of soluble compounds and because the anion is a single element.

The organism which was used in these studies was the *Bacterium coli* strain which has been used by Winslow and Falk (1918, 1918a) and by Cohen (1922). From its cultural and morphological characteristics it may be grouped as a *communis* type of *Bacterium coli* (sucrose negative, dulcitol positive).

In order that a wide range of chemical elements might be studied it was necessary to develop a rapid method for the determination of the number of bacteria per cubic centimeter.

[1] Presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Yale University.

It was decided that the simplest method for the estimation of bacteria was by a comparison of relative turbidities when the organisms were grown in a fluid culture medium, a procedure used with success by Holm and Sherman (1921).

It was necessary to provide a basic fluid medium with some buffer action and one which would be of sufficient complexity to produce a bacterial growth which would give measurable degrees of turbidity. For this purpose a one per cent solution of Bacto-peptone (Digestive Ferments Co.) was finally chosen. Ash determinations gave the inorganic content of the dried peptone as 4 to 5 per cent, so that a 1 per cent solution of the peptone contained about 0.05 gram of inorganic material for 100 cc. of solution. A communication from the Digestive Ferments Company stated that the ash was principally a sodium one, and that calcium oxide, phosphorus anhydride, sulphur and a small amount of chloride were also present.

Other media, of known chemical composition and lower ash content, did not prove useful as culture media. At first, growth studies were made with Dolt's synthetic media, composed of asparagine and di-basic sodium phosphate or asparagine and di-basic ammonium phosphate. These media were not sufficiently favorable to bacterial growth to produce marked changes in turbidity. A peptone mixture prepared by the hydrolysis of dialyzed edestin had a low ash content, but this product also gave insufficient bacterial growth.

In the development of a scheme of turbidity measurements it soon became apparent that accuracy could better be obtained through an average of many readings than by an attempt at mathematical precision through the use of turbidimeters or other optical devices. To estimate the growth, standard suspensions of bacterial cells, killed by heating at 56°C. for one hour, were used. The number of bacteria per cubic centimeter was determined by plating equivalent dilutions of the suspension of bacteria before heating. The standards were sealed with paraffin and kept at ice-box temperature; they did not deteriorate for a period of from six weeks to two months. Five sets of standards were prepared during the period of the study, each set with

approximately the same range of turbidities as determined optically and by the plate method. The relation between turbidity readings and bacterial numbers, recorded in millions of bacteria per cubic centimeter, are based on an average of the plate counts for the five sets of standards. Turbidities which are produced by more than 1200 million bacteria per cubic centimeter are difficult to read accurately and the figure 2700 was used to cover a general range between 2000 and 3500 million bacteria per cubic centimeter. The tables and charts in this article were prepared from figures obtained by averaging the turbidity readings of different tests.

With some of the less toxic salts a variation in the seeding of the tests might have caused a variation in the toxicity point; therefore it was necessary to inoculate with a uniform amount of culture. The stock culture of *Bact. coli* was grown in 1 per cent peptone solution. In inoculating the test fluids 1 cc. of a twenty-four-hour culture was added to 10 cc. of sterile water and with a pipette graduated to 0.01 cc. the culture was transferred in amounts of 0.05 cc. Thus in a single test each tube received the same amount of culture as its salt free control, although tests prepared at different times might have received slightly different amounts due to a variation in growth of the initial culture.

Efforts were made to use chemicals as free from impurities as possible. The sodium, potassium, calcium, barium and mercuric chlorides were purified by re-crystallization from distilled water. The ammonium, magnesium and strontium chlorides were Baker analyzed. The aluminium, cupric, lead, nickel and zinc chlorides were from Merck and Co. They were of 'C. P.' quality but were not re-crystallized. The cadmium, cobalt, ferric and ferrous, manganese and stannic chlorides were from Eimer and Amend, of tested purity with analyses. The cerium, lithium, thallium and titanium chlorides were from the same company, of 'C. P.' quality but not labeled with the analyses.

Salt solutions of known molar concentration were prepared, except in the case of titanium chloride, by adding carefully weighed amounts of salts to the proper amount of distilled water. The titanium chloride was supplied in solution, so that

the stock molar concentration was prepared from it by dilution. In all work on which conclusions are based anhydrous salts were used or the water of crystallization was allowed for in the calculations.

In order to obtain a series containing a constant 1 per cent peptone and varying molar concentrations of the salts, a solution containing 2 per cent peptone was used, from which by dilution with distilled water and stock salt solution the final product of the desired concentration was prepared. Calculated amounts of peptone solution and distilled water were added, by burette and pipette, to a series of bottles. The desired amount of stock salt solution was then added by pipette to one bottle and from this bottle the other solutions were prepared by dilution. The uniform results obtained tend to show that the solutions thus prepared did not vary appreciably.

Salts such as sodium and potassium chlorides and calcium and magnesium chlorides were added to the peptone solution as described, tubed in 5 cc. amounts and sterilized by autoclaving. Other salt solutions which could not be heated because of decomposition were prepared in the following manner: the stock salt solutions were made up and stored until they became sterile; the proper amount was then transferred with a sterile pipette to peptone dilutions which had previously been autoclaved. The solutions were then carefully poured into sterile test tubes calibrated to 5 cc. Contaminations occurred but rarely and were easily recognized by the appearance of a pellicle and by distinctly heavier growth. From all suspicious tubes plates were poured and microscopic examinations for spore-bearing organisms were made.

When added to the peptone solution, ammonium, calcium, lithium, magnesium, potassium or sodium chlorides caused no visible change. Salts which underwent hydrolysis with the formation of an insoluble hydroxide gave a precipitate in solution; and such solutions were shaken thoroughly before taking a sample with the pipette.

Barium chloride and many salts of the heavy metals gave a very troublesome precipitate when added to the peptone solution.

As the solutions could not be sterilized after preparation no attempt was made to filter the solutions and the lower concentrations were prepared by transferring a portion of the first prepared dilution, containing both solution and precipitate. In such cases the actual concentration of salt in solution in the final tube is unknown, except that it was not above the amount added.

Each set of test solutions consisted of four tubes of each dilution, three of which tubes were inoculated; and three inoculated tubes of a 1 per cent peptone solution were used as a control. The fourth tube of salt solution was used for an initial hydrogen ion determination.

The hydrogen ion content of the solutions was determined before inoculation and after growth had continued for ten days. The indicator method of Clark and Lubs was used. This method proved applicable even to salts which produced colored ions, as these salts were so toxic that the dilutions used were very high.

In the comparison of the test cultures with the standard bacterial suspensions direct and reflected light were used. Before comparison the tubes were shaken in order that any sediment might be distributed. Chemical precipitates did not usually interfere with the readings as growth did not ordinarily occur in concentrations where a precipitate was formed. There were some exceptions to this rule, barium chloride being especially troublesome.

To facilitate comparison with the standards a wooden block, painted black, with spaces for twelve test tubes in double rows was used. Rectangular openings, of about 0.5 cm. by 2 cm., extended from side to side of the block and permitted light to pass through the tubes. Behind the standard tube was placed a tube containing 1 per cent peptone solution and tubes of water were placed behind the test cultures to render the optical effect the same.

All tests of the growth of the bacteria in salt solutions were made at an incubation temperature of 37°C.

EXPERIMENTAL WORK

The salts studied were:

Sodium chloride	$NaCl$	Nickel chloride	$NiCl_2$
Potassium chloride	KCl	Thallium chloride	$TlCl$
Lithium chloride	$LiCl$	Cupric chloride	$CuCl_2$
Ammonium chloride	NH_4Cl	Ferric chloride	$FeCl_3.12\ H_2O$
Strontium chloride	$SrCl_2.6\ H_2O$	Ferrous chloride	$FeCl_2.4\ H_2O$
Magnesium chloride	$MgCl_2.6\ H_2O$	Zinc chloride	$ZnCl_2$
Calcium chloride	$CaCl_2$	Cobalt chloride	$CoCl_2 \cdot 6\ H_2O$
Barium chloride	$BaCl_2$	Lead chloride	$PbCl_2$
Manganese chloride	$MnCl_2.4\ H_2O$	Aluminium chloride	$AlCl_3$
Titanium chloride (15 per cent solution)	$TiCl_3$	Cerium chloride	$CeCl_3$
		Cadmium chloride	$CdCl_2$
Stannic chloride	$SnCl_4.5\ H_2O$	Mercuric chloride	$HgCl_2$

The salts divide into two general groups, one group with which no growth occurred in concentrations of 2 to 0.05 molar and a second group, where dilutions of 0.01 to 0.00001 molar were sufficient to prevent growth. As might be expected, those salts which are of common occurrence in the protoplasmic environment form the non-toxic group. They are the salts found in the first column above.

Sodium chloride may be studied as a typical mono-valent salt of group I. When the test cultures were examined at the end of twenty-four hours' incubation, growth was seen in all tubes which had a concentration of less than 1.0 M. The growth formed a fine evenly distributed suspension. There were no clumps in any dilution and when the tubes were shaken little or no sediment rose. A difference in turbidity existed in the different dilutions; those tubes which had a concentration of more than 0.25 molar showed less turbidity. The 0.25 molar concentration showed a relatively greater turbidity. At the end of two and three days' incubation the superiority of the 0.25 molar concentration for growth was even more marked, both higher and lower concentrations showing less turbidity than was observed in this solution. This gives a peak in the curve when the molar concentrations are plotted against the number of bacteria present as measured by turbidity. At the end of ten days this peak is obscured, for the organisms in the concentrations below 0.25

have usually multiplied until their turbidities equal those produced in the 0.25 molar concentration. The growth in the molar, 0.75 molar and sometimes the 0.5 molar concentrations showed a lag in comparison with the other concentrations. Those concentrations which showed a small amount of growth at the end of ten days contained little sediment, while concentrations which produced heavy growth showed more sedimentation.

Potassium chloride presented almost the same picture; and the curve of growth for ammonium chloride was the same. The growth picture was slightly different, here, as the organisms

TABLE 1

Average of readings for three days incubation period. Sodium, potassium, lithium and ammonium chlorides. Million bacteria per cubic centimeter

CONCENTRATION	NaCl	KCl	NH_4Cl	LiCl
4.0 M.	0			
3.0	0	0		
2.0	0	0	0	0
1.0	140	180	0	0
0.75	360	250	400	0
0.5	700	480	740	150
0.25	1700	1200	2700	500
0.125	1000	950	1100	600
0.05	1000	950	980	600
0.025	950	950	820	600
0.0125	820	950	700	450
0.005	850	950		
Control	900	800	500	500

showed a tendency to grow in clumps, and so to sink to the butt of the tube and form sediment. Lithium had not such a distinct optimum point but an optimum range between 0.25 and 0.05 M was noted.

If an average of the readings made at different concentrations after three day periods of incubation is taken, growth curves for the four salts are obtained which may be seen in table 1 and chart 1. The molar concentrations are the abcissae, plotted on a logarithmic scale against the turbidity readings as ordinates, plotted to the same scale. The heavy vertical line at the right

of the charts indicates a 1 molar concentration. The curves for sodium, potassium and ammonium show a rapid rise to an optimum at 0.25 molar concentration and a tendency to sink to a lower level of growth which is maintained, at this incubation period, in the remaining low dilutions. The optimum occurs at exactly the same concentration for all these salts.

All of these salts except ammonium chloride form solutions which are nearly neutral (pH 6.6–6.8). They are composed of elements which are strong bases and strong acids. The pH of the 2 M solution of ammonium chloride was 6 and that of all the less

TABLE 2

Average of readings for three days incubation period. Strontium, calcium, magnesium, barium and manganese chlorides. Million bacteria per cubic centimeter

CONCENTRATION	$SrCl_2$	$CaCl_2$	$MgCl_2$	$BaCl_2$	$MnCl_2$
1.0 M.	0	0	0	0	0
0.75		0	0		0
0.5	400	0	0		0
0.25	1450	1460	1130	0	0
0.1	1200	1530	2030	200	0
0.05	1570	2240	2200	800	0
0.025	1950	1500	1860	550	400
0.0125	700	1330	1700	400	400
0.005		1700			
Control	1250	1100	1160	550	700

concentrated solutions was 6.4. The peptone solution gave a pH of 6.8–7.0.

After ten days' incubation the concentrations which inhibited growth showed little or no change in hydrogen ion concentration. Tubes in which growth occurred, on the other hand, became very alkaline due to substances produced during metabolism. Thus the pH of the NaCl solution after growth was complete averaged 8.2; that of the KCl, 8.5; that of the NH_4Cl, 6.7; that of the LiCl, 7.8.

The bi-valent salts of group I were more toxic than the monovalent salts. Strontium chloride was the only one which ever

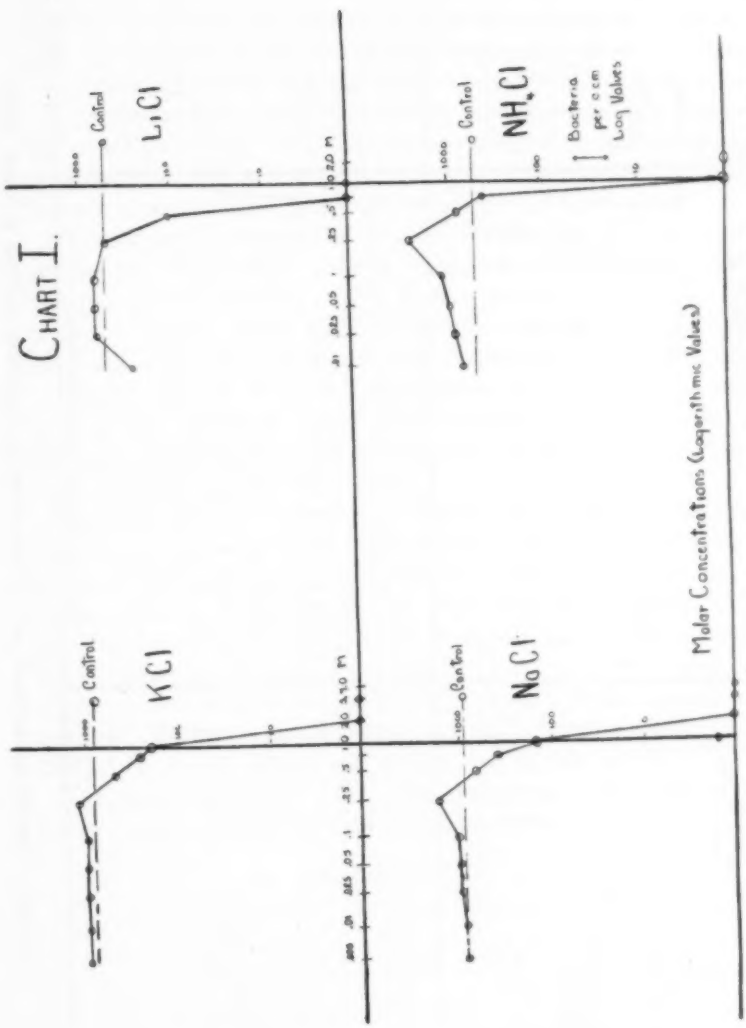

CHART 1. GROWTH OF *Bacterium coli* AS INFLUENCED BY SOLUTIONS OF FOUR MONOVALENT SALTS

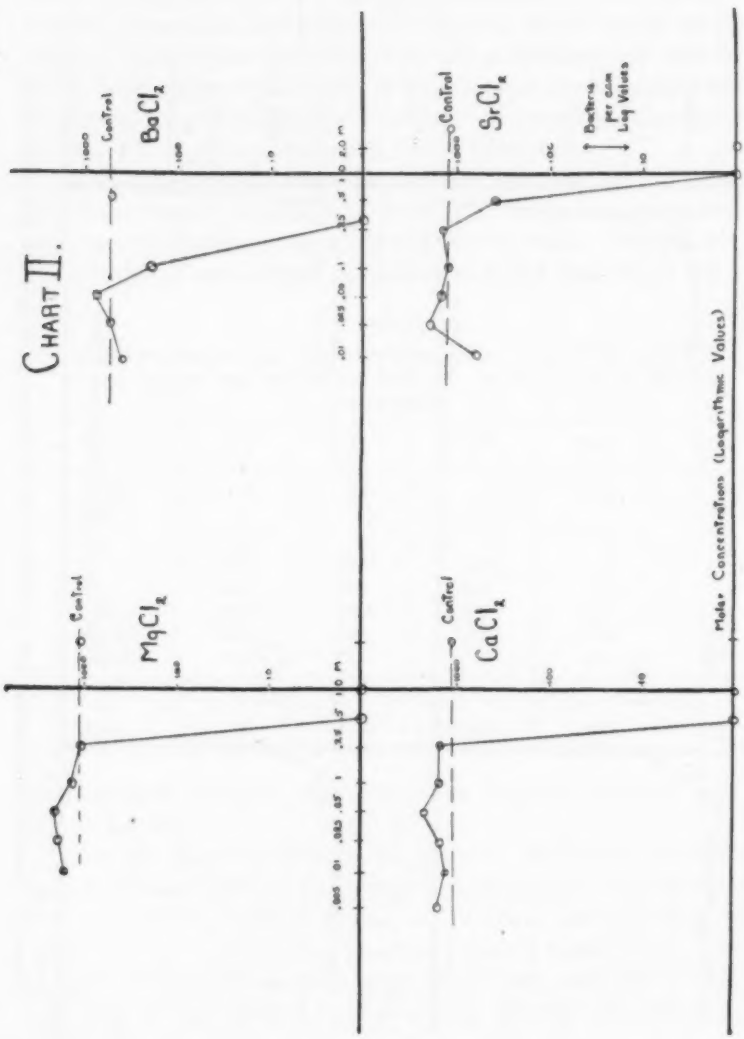

CHART 2. GROWTH OF *Bacterium coli* AS INFLUENCED BY SOLUTIONS OF FOUR BIVALENT SALTS

showed growth in 0.5 molar concentration and in two tests out of four there was no growth in this concentration. Barium, calcium, magnesium and manganese were all toxic in 0.5 molar concentration. With the exception of manganese an optimum concentration point was found where growth was better than in the peptone control. This optimum occurred in concentrations ranging from about 0.05 M to 0.025 M. The maximum point became obscured after an incubation period of three days.

There was a tendency for the bacteria to grow in clumps and at the end of ten days' growth there was much sediment with clearing of the solution. This was in marked contrast to the peptone control tubes and the tubes containing sodium and potassium where the organisms formed a homogeneous suspension.

Barium chloride formed a precipitate with the peptone solution in the dilutions used. The turbidity readings were, in this case, supplemented by observations on agar slants streaked with a loopful of the test solution.

The hydrogen ion concentrations were, in general, not different from concentrations found in solutions of the uni-valent salts of group I; although manganese showed a greater initial acidity in molar solution than any of the salts thus far reported. The pH was 5.8 rising to 6.8 at the 0.05 molar concentration, while the other solutions gave an initial pH 6.6–7.0. After the completion of incubation (ten days) those tubes in which growth had occurred showed the following averages: $SrCl_2$, pH = 8.2; $CaCl_2$, pH = 7.9; $MgCl_2$, pH = 7.4; $MnCl_2$, pH = 7.5.

The second group of salts studied comprises the salts of the heavy metals, which were found to be more toxic.

Group II

$AlCl_3$	$HgCl_2$
$CdCl_2$	$NiCl_2$
$CeCl_3$	$PbCl_2$
$CoCl_2$	$SnCl_4$
$CuCl_2$	$TiCl_3$
$FeCl_3$	$TlCl$
$FeCl_2$	$ZnCl_2$

Several conditions made this group difficult to study. The first was the difficulty of preparation, due to the fact that the

solutions could not be autoclaved. The method by which this difficulty was overcome has been discussed above; it provided a chance for contaminations, but such contaminations were easily recognized in the rare instances in which they occurred.

An examination of the hydrogen ion concentration data (table 3) shows very high acidity in even low concentrations of these salts, due to hydrolysis. The salts of group II are formed by

TABLE 3

Hydrogen ion concentration before incubation. Salts of the heavy metals. pH readings

CONCENTRATION	$AlCl_3$	$CdCl_2$	$CeCl_3$	$CoCl_2$	$CuCl_2$	$FeCl_2$	$FeCl_3$
0.01 M			6.0		4–5		2.0
0.005		6.4		3–4	4–5	4–5	
0.001	4–5	6.8	6.5	6.6	6.0	−6.0	4–5
0.0005	4–5	6.8	6.4	6.8	6.4	6.2	6.0
0.0001	6.6	6.8	6.6	6.8	7.0	6.4	6.8
0.00005		6.8	6.6	7.0		6.0	6.8
0.00001		6.8	6.6	7.2		6.8	6.8
0.000005		7.0	6.6				7.0

CONCENTRATION	$HgCl_2$	$NiCl_2$	$PbCl_2$	$SnCl_4$	$TiCl_3$	$TlCl$	$ZnCl_2$
0.01 M	5.0	4–5		4.4	4.4		+6.0
0.005	5–6	5–6	6.0	4.6	5.0	6.6	6.4
0.001		6.2	6.4	5.4	5–6	6.6	6.2
0.0005	6.6	6.4	6–7	5.6	+6.0	6.6	6.6
0.0001	6.6	6.6	6–7	6.2	6.8	6.6	6.6
0.00005	6.6	6.8	6–7	6.4			6.6
0.00001	6.6	6.8	6–7	6.8			6.6
0.000005	6.6	6.8	6–7				6.6
Peptone 1 per cent	6.8—7.0						

the union of weak bases and a strong acid; and in an aqueous solution the bases formed tend to be undissociated or insoluble. Such solutions have an acid reaction due to the presence of an excess of H-ions. Thus in the stronger concentrations these salt solutions had a visible inorganic precipitate and even in the weak dilutions there was always a portion of the salt which was not in solution.

When solutions of these salts were added to peptone solution there was again a tendency for precipitates to be formed, this time of an organic nature and due to the fact that a peptone fraction was thrown out of solution. This precipitate was often greater than that occurring in peptone solutions which were brought to the same hydrogen ion concentration with HCl and

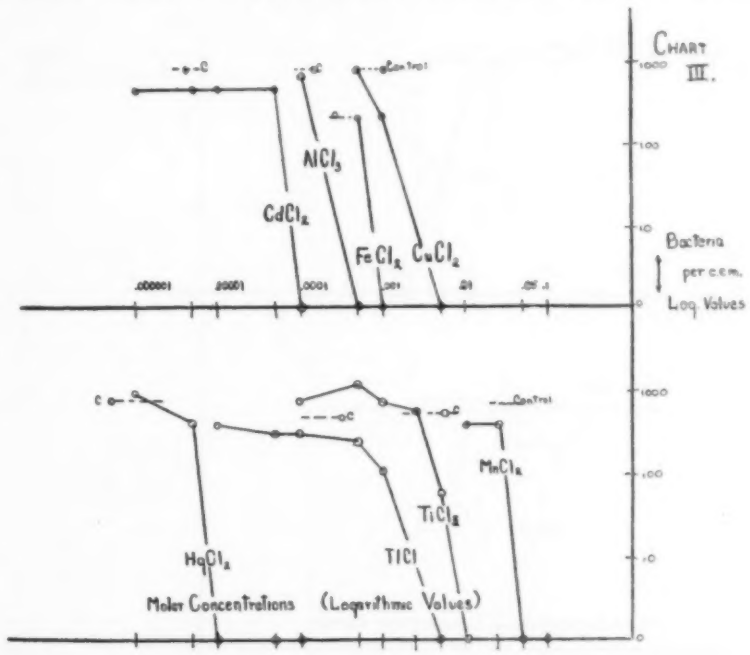

Chart 3. Growth of *Bacterium coli* as Influenced by Various Salts of the Heavy Metals

consequently must have been formed by action with the metal ion and not solely with the H-ions formed in hydrolysis. The work of many investigators tends to show that heavy metals which precipitate proteins, peptone or amino acids are themselves precipitated in so doing. In the formation of both of these precipitates some of the salt passes from the solution and we do not know the exact concentration of the final solution with which we were

working. In the preparation of tables and charts the molecular concentration as calculated in the preparation of the solutions is used but in any discussion of the results it must be remembered that a substantially smaller amount of salt was actually present in solution in the culture tube. Future work can define the toxicity points more closely by chemical analysis of the filtrates. The

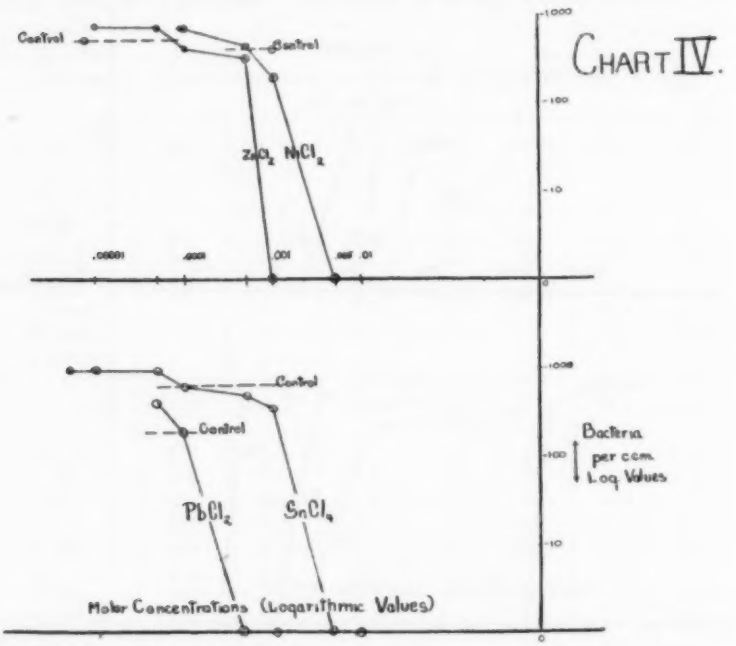

CHART 4. GROWTH OF *Bacterium coli* AS INFLUENCED BY VARIOUS SALTS OF THE HEAVY METALS

salts varied in the amount of precipitate formed in peptone solution. $SnCl_4$ caused the most marked precipitate of peptone solution per unit volume of salt, with $CeCl_3$ and $FeCl_3$ next, $AlCl_3$ and $BaCl_2$ third, $HgCl_2$, $PbCl_2$ and $TiCl_3$ fourth, and $CdCl_2$ $CuCl_2$, $FeCl_2$ and $ZnCl_2$ fifth, $CoCl_2$, $MnCl_2$, $NiCl_2$ and TlCl did not give appreciable precipitates.

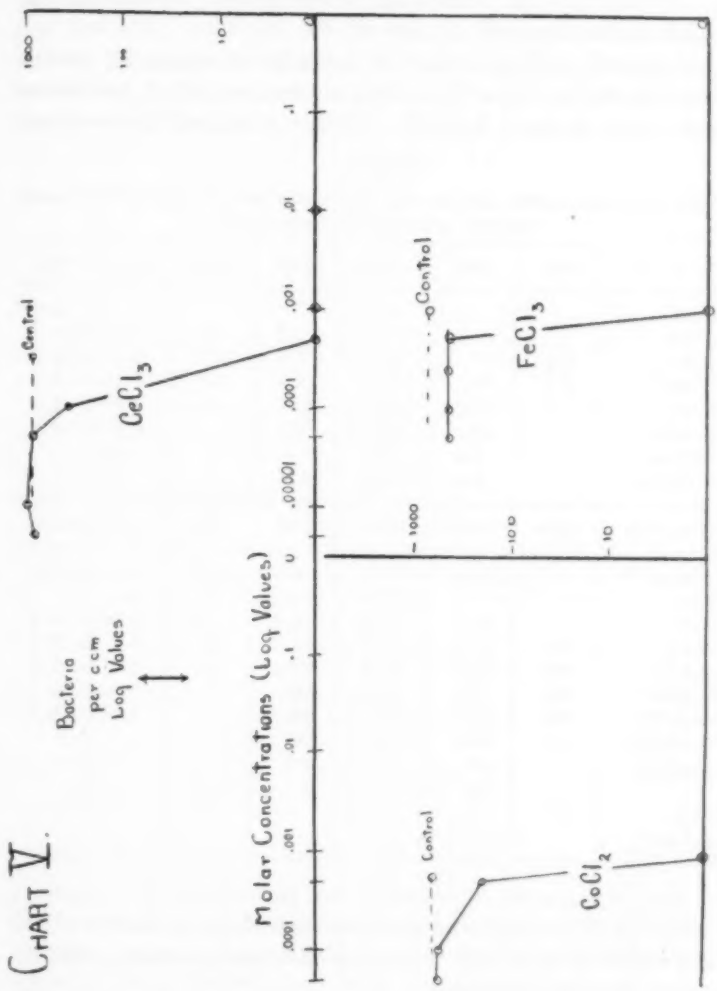

CHART 5. GROWTH OF *Bacterium coli* AS INFLUENCED BY VARIOUS SALTS OF THE HEAVY METALS

Table 4 gives the reaction after incubation and it will be noted that, as with the less toxic salts, where growth occurs the pH increases to between pH 8.0 and pH 9.0.

The varying toxicity points of the salts of group II, as determined at the end of a three days incubation period, are shown in table 5 and charts 3, 4 and 5. (Although its toxicity is noticeably less, manganese is included for convenience in charting.) Seven of the salts show stimulation of growth to an extent above that of the control, namely, cerium, nickel, lead, mercuric, stannic, titanium and zinc chlorides.

TABLE 4

Hydrogen ion concentration after ten days incubation period. Salts of the heavy metals. pH readings (averaged)

CONCENTRATION	$AlCl_3$	$CdCl_2$	$CeCl_3$	$CoCl_2$	$CuCl_2$	$FeCl_2$	$FeCl_3$
0.01 M			6.9				2.0
0.005				−5.0	4–5	4–5	
0.001	4–5		6.4	6.8	8.6	5–6	4–5
0.0005	5.4		6.5	8.4	8.7	7.1	8.3
0.0001	7.8	6.6	7.6	8.7	8.7	6.8	8.8
0.00005		8.9	8.6	8.7		6.8	8.8
0.00001		8.9	8.6			7.2	
0.000005		8.9	8.6				

CONCENTRATION	$HgCl_2$	$NiCl_2$	$PbCl_2$	$SnCl_4$	$TiCl_3$	$TlCl$	$ZnCl_2$
0.01 M		4–5		4.4	4.7		6.0
0.005		7.0	6.2	4.6	8.4	6.5	
0.001		8.0	6.5	8.4	8.6	6.2	6.4
0.0005	6.2	8.6	6.7	8.8	8.7	7.2	7.6
0.0001	6.6	8.8	8.5	8.6	8.7	7.6	7.8
0.00005	6.8	8.8	8.6	8.7		7.9	8.4
0.00001	7.0	8.8		8.7		8.6	8.7
0.0000005	8.8	8.9		8.7			
0.000001	8.8						
Peptone 1 per cent	7.8						

It seemed especially surprising that so toxic a compound as mercury should show this stimulation. The toxicity point was therefore checked by sterility tests made by streaking agar slants

with a loop of the culture. The lower dilutions did not show opalescence or any phenomenon which would interfere with the turbidity readings. No contaminations were found. The growth stimulation was not uniformly noted in the first days of incubation. It was most marked in the observations made after the ten day incubation period. The pH readings also testified

TABLE 5

Average of readings for three days incubation period. Salts of the heavy metals. Million bacteria per cubic centimeter

CONCENTRATION	$AlCl_3$	$CdCl_2$	$CeCl_3$	$CoCl_2$	$CuCl_2$	$FeCl_2$	$FeCl_3$
0.01 M			0				
0.005			0		0	0	
0.001			0	0	200	0	0
0.0005	0		0	200	700	200	550
0.0001	550	0	350	550			550
0.00005		450	700	550			550
0.00001		450	900				
0.000005		450	700				
Control	700	700	700	700	700	200	700

CONCENTRATION	$HgCl_2$	$NiCl_2$	$PbCl_2$	$SnCl_4$	$TiCl_3$	$TiCl$	$ZnCl_2$
0.01 M				0	0		0
0.005		0	0	0	60	0	
0.001		200	0	350	700	110	0
0.0005	0	450	0	500	1200	260	310
0.0001	0	700	300	600	700	330	400
0.00005	0	700	400	950		330	700
0.00001	0	700		950		430	700
0.000005	430	700		950			
0.000001	960						
Control	820	400	300	660	550	500	540

to increased growth for the peptone pH average was 8.3 and the average of the readings for the mercury solutions where increased growth occurs was 8.8 (see table 4).

The following experiment was undertaken to determine how far inhibition of growth by the salts of the heavy metals was due to the metallic ions themselves or to the hydrogen ion concentration.

Solutions of varying hydrogen ion concentrations were prepared by adding approximately N/10 HCl to the 1 per cent peptone solution. The hydrogen ion content was determined, as before, by the use of indicators and observations were made before and after sterilization. The peptone solution was sterilized in 5 cc. amounts and inoculated in the usual manner. Turbidity readings were made after varying incubation periods. The range of acidity unfavorable to this organism varied from pH 5.0 to pH 4.6.

An examination of tables 3 and 5 shows that for most salts of the heavy metals the pH is well beyond this unfavorable range in the highest dilutions at which growth is inhibited. In the case of three of the salts the free hydrogen ions may prove to be an important factor in toxicity.

	NO GROWTH	pH	GROWTH	pH
$SnCl_4$	0.005 M	4.6	0.001 M	5.4
$FeCl_3$	0.001	4.0-5.0	0.0005	6.0
$AlCl_3$	0.0005	4.0-5.0	0.0001	6.6

In these three salts growth occurred as soon as the dilution was used whose pH was favorable.

SUMMARY AND DISCUSSION

When grown in fluid culture media, bacteria produce a turbidity which can be used to estimate the number of bacteria per cubic centimeter. In these studies a 1 per cent peptone solution was used as a basic medium. Salts dissolved in the culture solution exerted an influence on the growth of *Bacterium coli* depending on the specific salt and the concentration in which it was added. There was some slight variation in effect at different incubation periods.

Each salt was so toxic at some concentration as to inhibit growth completely. The accompany table (table 6) shows the salt concentrations which thus limited bacterial growth. The group of the less toxic salts (termed group I in the preceding pages) includes the salts of the alkali metals and of the alkaline

earth metals. The toxic salts, or group II, consist of the salts of the heavy metals. It is seen that the salts of group I give neutral solutions and that, owing to hydrolysis, the salts of group II yield solutions with an acid reaction.

The results as to toxicity confirm, in general, those reported by Matthews (1904) for Fundulus, Woodruff and Bunzel (1909) for Paramoecium and Eisenberg (1916) for bacteria. The results of Eisenberg and the author in regard to relative toxicity parallel each other to a great extent. In 11 cases Eisenberg finds a

TABLE 6

Salt concentrations which limit bacterial growth. Incubation period, three days. Molar concentrations

SALT	NO GROWTH	GROWTH	SALT	NO GROWTH	GROWTH
$HgCl_2$	0.00001	0.000005	$MnCl_2$	0.05	0.025
$CdCl_2$	0.0001	0.00005	$BaCl_2$	0.25	0.1
$CeCl_3$	0.0005	0.0001	$CaCl_2$	0.5	0.25
$AlCl_3$	0.0005	0.0001	$MgCl_2$	0.5	0.25
$PbCl_2$	0.0005	0.0001	$SrCl_2$	1.0	0.25
$CoCl_2$	0.0005	0.0001			
			LiCl	0.75	0.5
$FeCl_2$	0.001	0.0005	NH_4Cl	1.0	0.75
$FeCl_3$	0.001	0.0005	NaCl	2.0	1.0
$CuCl_2$	0.001	0.0005	KCl	2.0	1.0
$ZnCl_2$	0.001	0.0005			
$NiCl_2$	0.005	0.001			
$SnCl_4$	0.005	0.001			
TiCl	0.005	0.001			
$TiCl_3$	0.01	0.0025			

higher molar concentration necessary for toxicity, in 4 cases the toxic concentrations are lower and in 6 the toxic concentrations are about the same as those of the author.

When the toxic concentrations are calculated in terms of molar solutions, the different salts may be arranged in a series of ascending toxicities as in table 7. The table shows, for each of the four workers (Matthews, Woodruff and Bunzel, Eisenberg and the author) a non-toxic and a toxic group of salts. The salts contained in each group are nearly identical, but in Matthews' work

the $AlCl_3$ and the $FeCl_3$ fall in the non-toxic group. Matthews found a great difference in the action of the bi-valent $FeCl_2$ and the tri-valent $FeCl_3$ which Eisenberg did not observe, and which did not appear in the present study.

TABLE 7

Relative toxicities according to four observers

MATTHEWS	WOODRUFF AND BUNKEL	EISENBERG	AUTHOR
$SrCl_2$	KCl	NaCl	NaCl, KCl
$BaCl_2$	$CaCl_2$	KCl	NH_4Cl
$MgCl_2$	$SrCl_2$	NH_4Cl	LiCl
$AlCl_3$		LiCl	
NH_4Cl	$MgCl_2$	$MgCl_2$	$SrCl_2$
KCl		$SrCl_2$	$CaCl_2$, $MgCl_2$
$CaCl_2$	$MnCl_2$	$CaCl_2$	$BaCl_2$
$MnCl_2$		$BaCl_2$	$MnCl_2$
LiCl	$CoCl_2$	$MnCl_2$	
$FeCl_2$	$CuCl_2$		$TiCl_3$
		HCl	
$CoCl_2$	$CdCl_2$		TlCl, $NiCl_2$, $SnCl_4$
$NiCl_2$	$NiCl_2$	$CeCl_3$	
		CrCl	$ZnCl_2$, $CuCl_2$, $FeCl_2$, $FeCl_3$
$ZnCl_2$	$PbCl_2$	$FeCl_2$	
		$FeCl_3$	$AlCl_3$, $CeCl_3$, $PbCl_2$, $CoCl_2$
HCl		$ZnCl_2$	
$CdCl_2$	$FeCl_3$	$ThCl_4$	$CdCl_2$
$AuCl_3$			
$CuCl_2$	$HgCl_2$	$AlCl_3$	$HgCl_2$
		$CuCl_2$	
$FeCl_3$		$TiCl_4$	
		$NiCl_2$, TlCl	
		$CdCl_2$	
		$PbCl_2$	
		$CoCl_2$	
		$AuCl_3$	
		$PtCl_4$	
		$HgCl_2$	

In 15 of the 23 chlorides studied a concentration was found which stimulated growth, as indicated by the production of a turbidity greater than that in the control solution to which no salt had been added (table 8). The stimulating salts included

not only K, Na, NH_3, Li, Sr, Mg, Ca and Ba but such toxic salts as those of Ti, Sn, Ni, Pb, Co and Hg. The stimulating concentrations for the latter were, of course, exceedingly low, (0.00005 molar in the case of Pb, 0.00001 molar in the case of Ce, 0.000001 molar in the case of Hg) while with K and Na a 0.25 molar concentration was stimulating. It is very possible that stimulating concentrations of the other 8 salts could have been established by more exhaustive study.

TABLE 8

Molar concentrations which stimulate bacterial growth. Three days incubation

SALT	CONCENTRATION	SALT	CONCENTRATION
NaCl	0.25 M	$TiCl_3$	0.0005 M
KCl	0.25	$NiCl_2$	0.0001–0.00005
NH_4Cl	0.25	$PbCl_2$	0.00005
LiCl	0.125–0.025*	$SnCl_4$	0.00005–0.000005
		$ZnCl_2$	0.00005–0.00001
$CaCl_2$	0.05	$CeCl_3$	0.00001*
$MgCl_2$	0.05	$HgCl_2$	0.000001*
$SrCl_2$	0.025		
$BaCl_2$	0.05		

* Growth stimulation slight.

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LE BACTERIOPHAGE DE D'HERELLE: SES APPLICATIONS THERAPEUTIQUES

A. BECKERICH ET P. HAUDUROY

Strasbourg, France

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Depuis l'époque ou d'Herelle présentait à l'Academie des sciences de Paris ses premières communications (1917), des expérimentateurs nombreux se sont mis à l'étude du phénomène qu'il a décrit. Nous le résumons en quelques mots: le filtrat d'un bouillon ordinaire dans lequel on met à incuber des selles de convalescent de dysentérie bacillaire ou de typhoide présente la propriété de lyser une culture jeune de bacilles dysentériques ou typhiques.

D'Herelle a très soigneusement étudié le fait qu'il avait mis en évidence, il en a abordé tous les aspects, a su construire une théorie de l'immunité par le bactériophage, apercevoir les applications thérapeutiques étendues qu'on pouvait en tirer. Nous allons, dans cet article, exposer, son hypothése sur la nature du principe bactériophage et sur quelles expériences il l'appuie. Nous rappellerons que d'autres hypothéses ont été émises, mais, sans prétendre à être complets.

Il est auparavant un point historique curieux à élucider. En parcourant systématiquement les revues scientifiques, on trouve que certains expérimentateurs ont vus le phénoméne avant lui, beaucoup ont eu entre les mains du bactériophage. Dans le "Traité de Microbiologie" de Duclaux on lit cette phrase "Rappelons les curieuses expériences de Hankin sur les eaux de la Jumma et du Gange, ou on voit une eau courante être aussi bactéricide qu'une solution de sublimé et perdre ce pouvoir après une courte ébullition sans qu'aucun précipité, sans que rien d'apparent, explique ce changement de propriétès. . . . ."(1898–1901). Hankin dans les Annales de l'Institut

Pasteur (1896) insiste sur cette action bactericide qui lui semble difficilement explicable.

Elle est à mettre au compte de la présence du bactériophage. Dans son livre "Le Bacteriophage" (1921), d'Herelle signale un autre fait du même ordre. Eliava, chargé d'examiner l'eau de la rivière Koura qui passe à Tiflis, y constate le phénomène suivant; s'il additionnait l'eau suspecte de peptone, après quelques heures d'incubation, on constatait la prèsence de très nombreux vibrions de forme normale; une douzaine d'heures plus tard, toute trace de ceux-ci avait disparu. Le phénomène resta pour lui inexplicable tant qu'il n'eut pas connaissance des communications concernant le bactériophage. En 1916 et 1917, Gildemeister écrit de longs articles dans le "Centralblatt für Bakteriologie" (1916–1917) pour décrire des figures de transformation du bacille dysentérique, du bacille typhique et du colibacille. Il étudie très complétement les colonies atypiques qu'il rencontre, et essaie de les classer. Le passage de colonies de formes atypiques à la forme typique le frappe; il insiste sur le fait que c'est au sortir de l'organisme qu'on rencontre de telles colonies. Les articles sont illustrés de photographies qui montrent que, sans aucun doute, Gildemeister a manié des colonies parasitées par du bactériophage.

Plus récemment Howard a signalé à la Societé de Biologie de Paris (1920) l'action lytique de certains sérums de typhiques: C'est là un phénomène que nous avons retrouvé et signalé (1922). Nous l'attribuons, pour notre compte, au bactériophage.

On voit, par ces quelques exemples, que le phénomène de la lyse en série de certaines bactéries, que les modifications apportées par le bactériophage dans l'aspect des colonies, ont été aperçus par les expérimentateurs. D'Herelle a retrouvé de semblables faits quand, à l'occasion d'une observation fortuite dans l'étude de la cocco-bacillose des sauterelles, il constata un arrêt inexpliqué de ses cultures. Il y revint plus tard, envisagea de nombreuses hypotèses avant de s'arrêter à celle qui lui parut concorder étroitement avec ses expériences.

Un grand nombre de bactériologistes avaient donc vu "le bactériophage," mais c'est d'Herelle qui l'a "découvert," nous

semble-t-il. On a beaucoup parlé, d'autre part, d'un phénomène décrit par Twort en 1915. Bordet, en particulier, (1921) veut y voir la lyse transmissible en série et il s'est fondé là-dessus pour contester à d'Herelle la priorité de sa découverte. Or la description donnée par Twort n'a que d'assez lointains rapports avec le bactériophage. Nous ne saurions entrer dans le détail d'une discussion, mais il nous parait probable que le phénomène de Twort et le phénomène de d'Herelle ne sont pas identiques.

Qu'est ce pour d'Herelle que le bacteriophage? Reproduisons la définition qu'il en donne lui-même dans son livre (Le Bactériophage, p. 10).

> C'est un petit être vivant, sans aucune présomption sur la règne auquel il peut être rattaché. Est-ce une bactérie, un protozoaire, un champignon? Je l'ignore, ses dimensions sont trop réduites pour qu'on puisse résoudre la question par l'observation directe au moyen des instruments optiques actuels. C'est une particule qui se développe aux dépens de la substance des bactéries, capable par conséquent d'assimilation, et qui est cultivable en sério in vitro sous sa forme filtrante.

Les expériences qu'il apporte à l'appui de sa théorie sont nombreuses, elles semblent exactes. Il est curieux de constater qu'un certain nombre de savants, ont émis d'autres hypothèses, sans soumettre à une vérification préalable maintes expériences fondamentales de d'Herelle.

Celui-ci base sa théorie d'un ultra-microbe sur quatre ordres de faits:

1. Transmissibilité indéfinite en série.
2. Numération possible des éléments bactériophages.
3. Adaptation du bactériophage à certains corps chimiques.
4. Polyvalence du bactériophage.

Examinons-les séparéments.

On peut transmettre en série, indéfiniment, le bactériophage. A partir d'un premier tube où la lyse s'est produite on transporte après chauffage ou filtration pour éviter les cultures secondaires, une goutte capillaire dans un second tube contenant une culture jeune du microbe lysable. La lyse se produit. Elle peut être

répétée aussi souvent qu'il plaira. C'est la reprise des expériences de Pasteur en vue d'obtenir des cultures pures et la preuve que le bactériophage est un principe apte à se reproduire.

La numération du bactériophage peut se faire soit en boulllon, soit sur gélose inclinée. En bouillon, par la méthode des dilutions, parmi les plus élevées, on note certains tubes où la lyse ne se fait pas, d'autres où elle est encore positive. Les premiers ne contenaient plus de bactériophage, les autres en avaient conservé un ou deux éléments susceptibles d'ailleurs de déclancher une lyse totale et de fournir, au titrage, un produit d'activité ègale à l'àctivité initiale: autre preuve de la reproduction du bacteriophage. Nous avons montré (1912) que la numération sur gélose inclinée est plus sensible que la méthode précédente. La présence du bactériophage se traduit par des "plages claires" petits trous réstés stériles au milieu de la nappe de culturé homogène. Ce seraient autant de véritables colonies isoléés de bactériophage et le fait qu'on puisse numérer un "principe," rend lui faire accorder une forme organisée.

Le bactériophage peut s'adapter à certains antiseptiques, la glycérol en particulier. En soumettant à l'évaporation une émulsion glycérinée de bactériophage, de manière à augmenter la concentration on retrouve à la fin de l'expérience le lysat vivant. Il périrait immanquablement dans une solution de glycérol aussi concentrée où il serait transporté d'emblée. On connaît des cas nombreux où les microbres s'accoutument aux antiseptique: "l'accoutumance est l'apanage exclusif des êtres vivants."

Le bactériophage est un: on entend par la qu'il possède une virulencé vis-à-vis de nombreuses bactéries. Quand on isole un bactériophage primitivement actif pour le coli-bacille, on peut l'amener à lyser le bacille typhique par exemple, il n'est pas invraisemblable que des passages suffisamment répétés puissent l'amener à lyser n'importe quelle bactérie.

Tous ces arguments constituent à l'hypothèse de d'Herelle un solide appui. Que lui objecte-t-on? En jetant un regard en arrière sur la masse des documents publiés, rien de probant! Personne n'a versé au débat un fait précis, une expérience décisive qui renverse les conclusions de d'Herelle.

Nous laisserons de côtè les théories de Kabeshima et de Salimben basées sur les experiences ou des constatations qui ne sont pas à l'abri des critiques. Bordet injectant dans le péritoine d'un cobaye du coli-bacille retire quelques heures après un exsudat contenant du bactériophage. Il échafaude toute une théorie: celle de l'autolyse microbienne transmissible, bien que les résultats des cette expérience soient irrégulièrs et que peu d'auteurs aient pu la répéter. La raison en est dans ce double fait: c'est le bactériophage préexistant dans l'intestin du cobaye qu'on retire à la faveur de la lésion minime produite par l'inoculation intrapéritonéale et au surplus la présence de ce bactériophage est irrégulière.

Lisbonne et Carrère ont émis successivement deux théories. Il ont d'abord fait provenir le bactériophage des leucocytes: cette première hypothèse fut infirmée par d'Herelle, (1922a, 1922b). Récemment, ils affirmérent que le simple jeu d'un antagonisme microbien suffisait pour faire apparaitre la lyse en série (1922). En mélageant du *Bacterium coli* et du bacille de Shiga, en filtrant les cultures obtenues et en faisant un certain nombre de passages, on verrait la lyse se produire. Nous croyons avoir montré qu'il étaient partis de cultures impures, de colonies modifiées parasitées par du bactériophage (1922).

Ces discussions sur la nature du bactériophage présentent un intérêt biologique considérable, mais un autre aspect de la question n'a pas été étudié avec l'ampleur qu'il mérite: c'est le chapitre des applications thérapeutiques, à peine ouvert à l'heure actuelle.

Dans sa première communication d'Herelle annoncait que son principe était capable de préserver le lapin contre une inoculation mortelle de bacille de Shiga. Ces premières expériences furent complétées par d'autres sur la typhose aviaire, le barbone. De la pathologie animale il est passé la pathologie humaine, a traité des dysentériques à l'aide d'ingestions de lysat. Les applications sont limitées, pour le moment, par le nombre encore peu élevé des souches actives qu'on possède: anti-dysentériques et paradysentériques, anti-typhiques et anti-paratyphiques, anti-staphylococcoque, anti-entérococcique, anti-bacterium-coli, anti-bacilli pesteux, anti-vibrion cholérique.

Résumons les résultats apportés par divers expérimentateurs et par nous-mêmes dans les diverses affections traitées. Une importante remarque préjudicielle s'impose: comme le bactériophage représente une médication nouvelle, les statistiques ne sont pas encore assez fournies pour décider s'il est le reméde spécifique de la dysentérie ou de telle autre maladie. Nous en sommes à la période d'éssai. Une précaution préalable consistera dans l'isolement du microbe en cause et sa mise en présence du bactériophage, qui le lysera ou non in vitro. On reconnaître par là les cas favorables, de ceux qui ne sont pas justiciable du traitement. Le mode d'administration le meilleur et la fixation des doses sont encore imparfaitement au point. Dans nos essais personnels nous avons tenté d'établir une posologie mais celle-ci varie en fonction de la virulence des souches.

LE BACTÉRIOPHAGE DANS LA DYSENTÉRIE BACILLAIRE

D'Herelle, dans son ouvrage (Le bactériophage, p. 211), rapporte sept cas de dysentérie traités par la seule ingestion de lysat. La plupart des malades étaient gravement atteints. 24 à 48 heures après l'absorption de bactériophage par la bouche, l'état général s'améliore, les selles sanglantes disparaíssent deviennent moulées et la convalescence s'établit dans les 2–3 jours. Ce sont là des résultats impressionants. Il paraît que Otto et Munter n'auraient pu les confirmer. Ne connaissant pas leurs observations nous n'en pouvons rien dire.

LE BACTÉRIOPHAGE DANS LA FIÈVRE TYPHOIDE

Au cours d'une épidémie de fièvre typhoide nous avons pu faire les premiers essais de traitement à l'aide du bactèriophage. Le 13 janvier 1922 nous apportions à la Société de Biologie les résultats suivants:

Deux cas chez l'adulte: formes ataxo-adynamiques; atteinte sévére du myocarde: (*a*) ingestion de lysat au 8^{ième} jour: crise de sueurs dans les deux heures; le surlendemain apyrexie d'une durée de 48 heures; (*b*) ingestion au 20^{ième} jour: pas de modification. Dans les deux cas, reprise et mort.

Deux cas, de typhoide chez l'adulte: formes ordinaires (*a*) ingestion au 18ième jour défervescence en lysis à partir du 20ième jour. (*b*) ingestion au 9ième jour, lysis à partit du 11ième jour. Crise de sueur dans les deux cas deux heures après l'ingestion. Convalescence.

Un cas de typhoide chez l'enfant: forme grave. Bactériophage par les deux voies au 20ième jour: Le surlendemain, l'apyrexie, nette et durable, succède aux températures élevées (un autre cas à association anaérobie parable n'a pas été influence).

Deux cas de para-typhoide B chez l'enfant: (*a*) Etat grave, bactériophage par les deux voies au 9ième jour, apyrexie nette et durable le lendemain, (*b*) forme ordinaire, administration au 23ième jour; apyrexie à partir du surlendemain.

Le résultats de ces 7 cas ne sont pas encore probants. Il semble bien que le bactériophage ait exercé une action favorable, mais nous pensons que le échecs sont dus, partie à une intervention trop tardive, partie à la faiblesse des doses administrées. Jusque là nous avions donné deux centimètres cubes au plus. Deux enfants atteints d'infection sévére sont traités par 5 cc. per os et 1 cc. sous la peau, l'un an 10ième jour, l'autre au 14ième Dans les deux cas, obnibulation et température élevée font place dans les 48 heures à la défervescence durable et à une euphorie marquée.

Un 3e cas, traité énergiquement chez un sujet de 24 ans, nous a procuré au 18ième jour, en 3 jours, une défervescence nette et durablé avec euphorie marquée (bien qu'au prix d'une réaction thermique un peu vive).

Si ces essais n'apparaissent pas comme décisifs, ils révèlent cependant une coincidence impressionante entre l'administration de lysat et une amélioration évidente. La réunion d'un grand nombre de cas apportera, croyons-nous, la preuve de l'efficcité du bactériophage dans le traitement de cette infection.

INFECTION À STAPHYLOCOQUES

Bruynoghe et Maisin ont été les premiers à appliquer le bactériophage dans les infections à staphylocoques (1921). Il se sont adressés à des malades atteints, soit de furonculose,

soit d'anthrax. Aussi près que possible de la région malade ils injectent 0.5 cc. à 2 cc. de lysat. L'empâtement diminue rapidement et les lésions disparaissent en 24 ou 48 heures.

Gratia (1922) a publié les résultats obtenus par lui. Dans les cas d'abcès et de furoncles le processus de guérison est notablement accéléré par l'administration de lysat. Le pus est évacué rapidement parfois même se résorbe sans laisser de cicatrices. Nous avons, de notre coté, tenté des essais qui seront publiés quand nous estimerons avoir réuni un nombre suffisant d'observations pour pouvoir fixer la posologie et le mode d'administration de ce mode de traitement. D'ores et déjà nous indiquerons qu'au lieu d'un bactériophage anti-staphylocoque doré unique, nous utilisons un mélange de bactériophages spécialement et séparément exaltés: anti-staphylocoque-doré, anti-staphylocoque-blanc et anti-entérocoque. Nous ménageant ainsi plus de chances d'arriver à un résultat favorable au cas d'une infection polymicrobienne éventuelle. Nous prenons toujours soin (ici encore) de faire un essai de lyse in vitro du microbe isolé. Cette précaution nous a d'ailleurs conduits à la constatation que les germes isolés d'infections chroniques sont fréquemment résistants à la lyse.

Nos résultats, d'une manière générale, concordent avec ceux de Bruygnoghe et de Gratia.

En somme, si les trauvaux accumulés n'ont pas indiscutablement mis en lumière la nature du bactériophage, il n'en importe pas moins de s'engager sans tarder dans la voie, qui promet d'être fertile, des applications thérapeutiques étendues et variées.

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ON THE VARIATION OF BACTERIUM COLI

HILDING BERGSTRAND

From the Department of Pathology of the Royal Caroline Institute, Stockholm

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INTRODUCTION

Before the time of Koch bacteria were supposed to be capable of a very great variation. But the plating method showed that the supposed variants were usually contaminating microörganisms. As so often happens, the new discoveries and the theories based on them were over-emphasized and bacteriologists went to the other extreme. Of course a great many articles were written about bacterial variation, but they were never taken into consideration. Even the striking discovery of the *Bacterium coli mutabile* by Neisser and Massini (1907) did not attract any very general interest. Everybody who has read, for example, the modern literature on the types of pneumococci and meningococci, etc., must admit that there is very little talk about variation of the types. As far as I can understand, the types are supposed to be constant. A pathogenic type can change its virulence, but it is not to be confused with an apathogenic one.

In the case of pneumococcus the distinction between the types is made only by the agglutination test. They do not differ morphologically or culturally. It is then justifiable to ask if it is not possible that one type can change into another by losing or acquiring agglutinability, erroneously regarded as specific. As a matter of fact this is already shown to be the case with *Bacterium coli*. Baerthlein (1912) obtained from a pure culture of *Bacterium coli* two types, one agglutinable not only by its own antiserum, but also in high dilution by the antiserum of the other type, which, on the contrary, was very slightly agglutinable by either serum. And neither of these types was constant, one

after a while passing over into the other. These discoveries have recently been partly confirmed by Bordet and Ciuca (1920) and Gratia (1922) in their works on the Twort-d'Hérelle phenomenon.

Certainly the problem is of interest and involves questions of great importance. One may ask: does a variation of a non-pathogenic type into a pathogenic one often happen, and under what conditions? Does the isolation of the patient really compensate for the effort and the expense under such circumstances? It is obvious that the answers to these questions will be very different in the case of different bacteria. We know that a satisfactory isolation against *Spirochaeta pallida* ought to check syphilis, but we are not sure of the result in the case of diphtheria and still less in pneumonia. There is justification for the assumption that an apathogenic pneumococcus can change into a pathogenic one and give rise to an epidemic or only to some single cases. It is also possible that this epidemic may disappear, not only because the individuals still living have acquired immunity, but also because the microörganisms are inclined in time to change their type. Topley's (1921) remarkable observations in spontaneous epidemics among white mice point in this direction. He showed that the microörganisms and the living animals established a modus vivendi and the epidemic died, but when new animals were mixed with the old a new epidemic occurred, affecting not only the new but also the old "immune" individuals.

The above-mentioned facts are sufficient, I think, to demonstrate that the variation of bacteria is well worth more general interest. Fortunately the recent discovery of the Twort-d'Hérelle phenomenon seems to give these studies new life.

In the investigations on bacterial variation, related in the following pages *Bacterium coli* is used as an object for several reasons. This bacterium has been worked with on these lines by many previous authors and it seems to be subject to variation in a specially high degree; it is easy to cultivate and harmless to handle.

1. Isolation of the types

An old laboratory culture of *Bacterium coli* was seeded in broth and left for a fortnight and then plated. The plates showed two kinds of colonies, which were called D (dull) and S (shining). The D-colonies were large, non-transparent, yellowish, with an uneven stripy border and a dull slightly corrugated surface; the S-colonies were small, transparent, bluish, sharp-bordered and showed an even shining surface. The populations of the cultures also differed morphologically, the D-type consisting mostly of long slender individuals and the S-type of short clumsy ones. On fishing, the D-colonies turned out to be dry and cracky, the S-colonies, on the contrary, moist and of a paste-like consistency. This difference is demonstrated in figure 4. Both types possess the general properites of *Bact. coli* with regard to morphology, the sugar fermentation tests and the indol production test. They are also agglutinated by several coli sera, as will be mentioned later on. The D-type is not motile; the S-type on the other hand is very motile. In a twenty-four-hour-old broth-culture (pH 6.8) the D-type produces a supernatant film and a sediment on the bottom of the tube; the broth between the film and the sediment is clear. The tubes seeded with coli S show a homogeneous turbidity and a whitish band at the level of the fluid surface, soon developing to a film. Later a sediment is to be seen also in these tubes. But a difference persists for weeks between the broth-cultures of the two types. The broth never becomes clear in the S-tubes and the sediment is cloudy with an uneven surface. The sediment in the D-cultures has a smooth surface just like sand in the bottom of a glass of water (fig. 10). It is evident that the type S corresponds to one of the types observed with *Bact. coli* by Gratia and described as type R. He found on old agar slants in the dull film small vitreous colonies containing a pure culture of a type differing greatly from the organisms of the original strain. This new type was very resistant against the lytic agent, more virulent and less phagocytable than the original type, and had a lively motility. On the other hand he observed a non-motile sensitive and aviru-

lent type, that evidently is identical with our type D. Baerthlein also talks about opaque (trüb) and non-opaque (hell) colonies.

After repeated plating isolation was made *ad modum* Burri in order to obtain cultures of as great a purity as possible. I am of course aware of the fact that no certain method of obtaining single cell cultures exists. It is possible however, to keep the cultures pure for months by daily plating and their general behavior in the experiments affords sufficient evidence that these first cultures can be accepted as derived from a single cell or at least from a couple of identical cells.

EXPERIMENTAL

Experiment 1. A loopful was taken from an eighteen hours slanted agar culture of each type and spread out in a test-tube containing 5 cc. plain broth of a hydrogen concentration of pH 6.8. The bacteria of the D-culture were very difficult to spread. At last, however, a homogeneous suspension seemed to be obtained not only of the S-type, which easily mixed with the broth, but also of the D-type.

From these two suspensions two equal series of tubes containing broth of decreasing hydrogen concentration were seeded, each tube with 0.5 cc. Kept at room-temperature (18°C.) for eighteen hours a loopful was removed from the culture and thoroughly mixed with 5 cc. plain broth of the same above-mentioned hydrogen concentration. From each of these dilutions in turn a loopful was spread on agar-plates, incubated for eighteen hours, and then examined. The number of the colonies and the pH values of the corresponding tubes are indicated in table 1. This first experiment was carried out on February 13 to 15. Afterwards samples were removed and treated in the same way on February 18, February 25, March 5 and March 14 in order to study the supposed appearance of variants and their behavior. The results of these platings as to the numbers of colonies are recorded in tables 2 to 5. By examining the first series of plates a striking lack of parallelism between the size and number of the D-colonies was the most outstanding feature. The D-colonies were several times larger

TABLE 1

February 15

	NUMBER OF TUBE							
	1	2	3	4	5	6	7	8
pH	4	5.1	5.9	6.6	7.2	7.6	8	8.4
Type S	0	1	71	67	59	84	60	91
Type D	0	0	19	22	26	19	21	25

TABLE 2

February 18

	NUMBER OF TUBE							
	1	2	3	4	5	6	7	8
pH	4	5.1	5.9	6.6	7.2	7.6	8	8.4
Type S	0	190	1200	1400	1400	1200	1100	1300
Type D	0	5	43	46	110	90	84	115

TABLE 3

February 25

	NUMBER OF TUBE							
	1	2	3	4	5	6	7	8
pH	4	5.1	5.3	6.6	7.2	7.6	8	8.4
Type S	0	500	500	600	800	500	500	600
Type D	0	100	78	80	110	150	250	200

TABLE 4

March 5

	NUMBER OF TUBE							
	1	2	3	4	5	6	7	8
pH	4	5.1	5.9	6.6	7.2	7.6	8	8.4
Type S	0	500	150	200	150	200	200	200
Type D	0	31	150	150	250	250	150	250

TABLE 5

March 14

	NUMBER OF TUBE							
	1	2	3	4	5	6	7	8
pH	4	5.1	5.9	6.6	7.2	7.6	8	8.4
Type S	0	150	110	95	120	90	140	63
Type D	0	120	150	200	225	65	110	200

in size than the S-colonies but the latter were much more numerous. Granted that the organisms are of the same shape and that the difference is not due to an excess of culture-medium in the D-plates, this fact must be regarded as a contradiction. That the D-colonies really grow more rapidly than the S-colonies on agar-plates was shown by making plates containing the same numbers of colonies spread regularly over the plates. Furthermore, the D-microörganisms certainly differ in size from the S-microörganisms, but this could not be accepted as a sufficient explanation. In order to solve the question a new experiment was carried out.

Experiment 2. As it was necessary to obtain a bacterial suspension of as exactly known a density as possible, a broth-culture eighteen hours old was thoroughly shaken for one-half hour, diluted and then mixed with a suspension of washed red blood-corpuscles. Examination of a few samples now showed that it was very easy to get the S-microörganisms isolated but almost impossible to separate the D-bacteria sufficiently. Thus it was immediately clear that the great size of the D-colonies is due to this fact; they do not originate from single cells, but, at least mostly, from clusters of cells. This will also be recognized from the following experiment, revealing at the same time a new phenomenon.

Experiment 3. In order to obtain a good suspension and at the same time to study the effect of a most thorough shaking, a broth-culture of each of the two types was first shaken during five minutes and a sample removed and spread on agar, then left one-half hour and afterwards shaken again with glass-beads during one-half hour and an equal sample plated. Originally it was intended that the clusters should sink to the bottom, thus making it possible to obtain a homogeneous suspension in the fluid above. But the D-tubes showed already when examined macroscopically a poor suspension. Hence the second shaking was made. Plating now showed the remarkable fact that the D-colonies had decreased and the S-colonies increased in number. Thus the shaking with the glass-beads had caused an agglutination of the D-microörganism, which could be confirmed by

direct microscopical observation. On the other hand the S-bacteria had been separated in a higher degree by the second shaking than before. The details are indicated in table 6.

These findings are of importance in considering the above-mentioned plating experiments and the agglutination tests described below. They show that even when the colonies look as if they had originated from one single cell this is not at all certain, and that the plating method has only a relative value in counting the living microörganisms in a broth-culture, at least as far as *Bact. coli* is concerned.

Experiment 4. As is to be seen in tables 1 to 5, the limiting pH values on the acid side lie between pH 4 and pH 5.1. and seem to be the same for both types. In the first tube, of the highest hydrogen ion concentration, not only does no growth occur but the microörganisms are killed and this occurs, as a new

TABLE 6

TYPE	D	S
Numbers of colonies after the first shaking	42	76
Numbers of colonies after the second shaking	13	275

experiment showed, in thirty-six hours for the S-type and in twenty hours for the D-type. In the second tubes too at first many bacteria die and it is only after an interval of several hours that a part of them recover from the injuries and begin to multiply. This is shown by the fact that plates seeded with a loopful from control-tubes containing 5 cc. broth inoculated immediately before with one-half cc. of the original bacillary suspension show from five to ten colonies. In the second tubes the growth is delayed to a very considerable degree but rises in time to the same level as in the other tubes. The decrease of the living microörganisms in the cultures occurs earlier in the S-tubes and is more marked. It appears that the number of viable bacteria for long periods is fairly the same in both S-tubes and D-tubes with perhaps an increase in later tubes as shown in table 5. Considering the peculiarities mentioned in experiment 3 it is, however, necessary to be careful that this is

not due merely to a higher degree of suspension of the bacteria. On the other hand there appeared in the D-tubes a new phenomenon which, as we shall see later, is perhaps responsible for these figures. In order to obtain the limiting hydrogen concentrations with as great accuracy as possible two new series were seeded and examined in the same way as before, except that the plating was made twenty-four hours after the seeding of the tubes. The results are indicated in table 7. The method used for preparing the broth-tubes was the following. Two litres of broth are obtained by boiling 1 kgm. minced ox meat for one hour and adding 20 gram pepton Witte and 10 gram NaCl. The broth divided in small portions is adjusted by NaOH and HCl to the desired pH, filtered into tubes and sterilized. Afterwards the tubes are tested again and suitable sets taken for use. The test

TABLE 7

April 8

	NUMBER OF TUBE														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
pH	4.3	4.8	5.0	5.2	5.8	6.3	6.8	7.2	7.6	8.2	8.6	9.0	9.2	9.3	9.4
Type S	0	0	62	400	500	500	500	500	600	600	100	42	65	17	0
Type D	0	0	12	36	42	250	250	250	300	250	45	0	0	0	0

of the hydrogen ion concentration was made by the colorimetric method and the indicators used were those of Clark and Lubs, kindly provided by K. G Dernby.

This experiment evidently confirms the above mentioned observation that a too high hydrogen concentration delays the growth of the type D more than the growth of the type S. Or in other words the "lag" is greater in type D. The "lag" is very well marked up to the D tube 6, while type S multiplies practically just as rapidly in tube 4 as in tube 8. On the alkaline side too, the type D is more sensitive. A pH value of 8.6 gives a marked "lag" and a pH of 9.0 kills the microörganisms, as samples removed some days later showed. On the other hand type S shows a great resistance, multiplying up to a pH value of 9.3; and control-tubes showed that a number of the seventeen colonies were capable of growth.

Experiment 5. The tubes were left for a month and then examined. Now it turned out that the "lag" was not the only injury that the high and the low hydrogen ion concentration caused. It may be true that the tubes at the ends of the series also got their chance and for a time developed as rapid a growth as the other tubes, as is indicated in the series indicated in tables 2 to 5, but a comparison of the sediment in the different cultures shows that the sum of growth is far less in these delayed tubes.

The sediment having been measured, the tubes were filtered and the filtrates tested for pH. The examination showed that the hydrogen ion concentration had decreased in the tubes on the left side and increased on the right side. The pH value was much alike in all tubes oscillating only between 6.8 and 7.2 approximately. The microörganisms evidently had readjusted the hydrogen ion concentration to their own benefit in a very remarkable way. An exception on the acid side was noted in tubes 1 to 3 of both series. This is quite natural for tubes 1 and 2 but surprising in the case of tube 3. The difference in hydrogen ion concentration between tube 3 and 4, slight in the non-seeded broth, was in the filtrates really striking, when we recollect that tube 3 also developed an appreciable growth. The remains of the cultures at the ends of the series were now seeded in plain broth and this experiment showed that the cultures of No. 3 were dead. Thus it seems as if the bacteria in these tubes died because they were unable to make a growth strong enough to readjust the medium to a hydrogen concentration necessary for a longer life. That the first series did not reveal this phenomenon may be due to the higher pH value (5.2) in the first tube capable of growth in these series.

No corresponding phenomenon was observed on the alkaline side where only the tubes without growth were unchanged.

In the preceding notes we have chiefly considered the numbers of colonies observed on the agar-plates seeded from the broth-cultures but these themselves show several features worth mentioning. I only record the findings in the series of April 8, since they do not differ from the observations made in the first experiments. The D-tubes 6, 7, 8, 9, 10, kept at room-tempera-

ture (18 cc.) for twenty-four hours, all show a sediment of the same thickness and a clear fluid above: in the eleventh tube no growth is visible; tubes 3, 4, 5 are slightly turbid but without sediment. The S-tubes 4, 5, 6, 7, 8, 9, 10 are very turbid and tubes 12, 13, 14 are slightly troubled, all without sediment; tube 3 shows a small sediment and a clear fluid. Apparently the D-cultures show the character of an S-culture and conversely in the tubes approaching the limit of the hydrogen concentration on the acid side, but both keep their character on the alkaline side. In the D-tube 11 the broth is clear and the growth invisible, since the sediment is not yet heavy enough to be recognized. Thus the behavior of a type of *Bact. coli* in a broth-culture is characteristic only at a fixed hydrogen ion concentration. Spread on agar the cultures show no difference. Evidently we are not dealing with any hereditary fixed modification.

After forty-eight hours a considerable sediment has developed in the S-tubes 4, 5, 6, 7, 8, 9, 10 and a less marked one in the third and eleventh. The other S-tubes and the D-tubes have practically the same appearance as before. Already during the first twenty-four hours a whitish band has appeared in the S-tubes adhering to the glass wall at the level of the fluid surface anf from this band a supernatant film developed during the next day. At the same time that the whitish band became visible in the S-tubes the first traces of a supernatant film appeared in the D-tubes. The film reached in a short time a much stronger development in the D-tubes than in the S-tubes. My observations in this matter are not in absolute agreement with those of Gratia, who writes that his R-type (corresponding to my S-type) multiplies slowly and produces a film later. As is indicated by the above-mentioned experiments, it is, however, very difficult to judge which type really multiplies most quickly. In the following days the film sinks to the bottom and the cultures assume their definite features described above and demonstrated in figure 10.

DESCRIPTION OF THE VARIANTS

Shortly after the first definite isolation of the types both were seeded on agar-plates in such abundance that on the S-plates an uninterrupted film soon developed. On the plates of the D-type no confluence occurred. A month afterwards the D-plates showed a great many small secondary colonies (fig. 2), resembling transparent shining droplets. Evidently these secondary colonies correspond to those described by Massini with *B. coli-mutabile* and more elaborately dealt with by Nyberg (1912) under the name of "daughter colonies." Fishing from these colonies did not produce any other colonies than the common dull type, when streaks were made on agar-plates either directly or after dilution in broth. The S-plates (fig. 1), on the other hand, showed very few secondary colonies and of an entirely different character, having the form of rather big rings, growing up a little over the surface of the film. It was easy to obtain by fishing from these rings a new kind of colonies mixed with colonies of the old type, as is clearly to be seen in figure 5. The new colonies were whitish, shining and opaque and did not resemble either the S-type or the D-type. On replating, this type disappeared, giving rise to two new kinds of colonies, one forming rings with a whitish shining surface and surrounding a transparent bluish tiny central disc (fig. 12), another kind dull, cracky, opaque and in many other respects resembling the D-type. These were called SD (fig. 12). Subcultures of the new ring-forms in their turn produced SD-colonies. Thus the above-mentioned secondary types were apparently only transient except for the SD-type which turned out to be very constant. Repeated transfers in broth even at long intervals did not change the type nor was it possible to get the old forms back or to obtain new ones by keeping the plates for months. When, as will be noted later, the agglutination test also showed a resemblance between this new type and the old D-type, it was justifiable to suppose that the S-type had changed into a D-type. But an examination of the broth-culture in the hanging drop showed that the SD cells were motile. We may now, however, leave these plates for a moment.

As will be recalled, two series of broth-cultures were seeded on February 13 and samples removed and spread on agar-plates. The cultures on these plates did not show any aberrant types. The next time the broth-cultures were tested was on February 18. The S-plates were still unchanged, but every one of the D-plates showed a few shining sharp-bordered transparent colonies resembling the S-type. Sub-cultures in broth gave turbid growth. But in the hanging drop it was impossible to find one single motile microörganism. Thus the new type, being after all not a true S-type, was called DSh. At every new test the D-tubes showed such colonies in increasing number and the last time, on March 14, they were at least equal in number to the old colonies. The S-tubes on the contrary showed a great uniformity and the first variants were seen on March 5. These new colonies were in every respect identical with the above-mentioned type, SD, obtained from the secondary colonies of the old agar-plates of the S-type. Here we can now leave the S-type, since it did not show anything more of interest.

As for the D-type one of the plates showed on February 25 a phenomenon which was at first regarded as due to a contamination. Some of the dull colonies showed transparent fluid spots which in time spread over the whole colony and totally dissolved it. When the plate was tilted, the colonies of this type flowed to the lower level as a heavy fluid (fig. 11). The next test, on March 14 showed a few colonies of the same appearance in two other tubes and furthermore many of the dull colonies, at first of normal character, produced in two or three days on the border of the colony a fluid zone, as is to be seen in figure 9. At this time a new paper of Gratia on the Twort-d'Hérelle phenomenon appeared, in which he describes a mucoid type of *Bact. coli* obtained by treating the cultures with the lytic agent. His pictures and descriptions afford, I think, sufficient evidence that we are dealing with the same varient of *Bact coli;* it appears, however, that in my case the mucoid quality is exceedingly pronounced. I venture to say this because, when transferred daily from tube to tube during a long period, the fluid character of the colonies gradually decreased and at last totally disappeared

in some tubes. Thus there is every degree of mucoid growth. It was interesting to see that on the disappearance of the mucoid growth the same dull colonies with a mucoid border-line appeared as in the beginning.

Evidently we are dealing here with a variant of *Bact. coli*. As it dissolved the old growth, a filtrate was tested but showed no influence on a culture of Type D. When kept for some days the mucoid growth dries up and can no longer be transferred to other cultures. Fishing from such plates gives a pure culture of dull colonies. On slides the mucoid substance is easily stained with gentian violet and even with methylene blue and then looks like mycelioid threads; in the hanging drop nothing but the bacteria is to be seen. As will be seen later this variant is very interesting because it is inagglutinable.

Finally one other variant may be mentioned, appearing in one of the D-tubes after the plating on March 14. On one plate were observed some tiny colonies, which, transferred into broth, showed slow growth, making the fluid turbid. Samples removed and spread on agar again gave a tiny growth of very irregular colonies, as is to be seen in figures 6 and 7. After a while, however, a luxuriant secondary growth appeared as demonstrated in the above mentioned figures. The difference between the tiny initial growth and the secondary luxuriant vegetation is, however, best visible in figure 3, which is a photograph of a slanted agar tube seeded from the original tiny colonies. Only two colonies have developed from the abundant bacteria that were seeded and for more than a week they gave only a scanty transparent vegetation, when at last a new fresh growth appeared in one of the colonies as a surrounding ring and in the other as a ring and a central heap. The behavior of the colonies reminds one strongly of the Twort-d'Herelle phenomenon. All attempts, however, to obtain an active filtrate failed.

Finally one may ask how long these variants are stable. In order to answer this question pure cultures of the variants DSh and SD were seeded in plain broth and samples tested on plates every eighth day. They are now more than two months old and both have acted in the main in the same way. Three

groups of colonies of different kinds are to be seen: (1) pure colonies of the variant itself; (2) pure colonies of the mother type, and (3) several colonies of new types not analyzed.

Plates kept for weeks produce, when replated, pure cultures. By transferring from tube to tube every second or third day it is also possible to keep the cultures pure in broth. Evidently it is easier to keep a type pure on agar-plates than in broth. That even on the plates a variation occurs has, however, been demonstrated above.

I have spoken above about pure cultures of the different types, although I am aware that the purity never can be absolutely sure. On the contrary it is very likely that the colonies of the D-types at least generally include some suppressed individuals of several variants. The impossibility of obtaining variants by fishing from the secondary droplets on the D-colonies indicates that this is probably the case. Nevertheless this must not be taken as an objection to the theory that a single cell is able to produce a growth of several variants.

By our experiments the D-type has been transferred to an S-type and conversely, except for the motility. The motility is, however, not a stable quality, as Gratia has shown. Thus there is no doubt that both the types of *Bact. coli* are only relatively fixed. This is extremely interesting, since, according to Gratia, the two types differ in virulence. In all our experiments the hydrogen ion concentration did not seem to be of any great importance for the variation, since, with one exception, the variants were the same in all tubes and occurred approximately at the same time.

AGGLUTINATION TESTS

Several agglutination tests were made, but it is not necessary to record more than one of them, since they do not differ in principle. The tested strains were one original pure D-type called D, one original pure S-type called S, one dull S-type called SD, one shining D-type called DSh, one mucoid type derived from a D-culture and called DF, and finally the above-described tiny strain called DT and its secondary luxuriously growing

strain called DTL. The bacteria were cultivated for eighteen hours on slanted agar and washed off by saline solution, mixed with the serum dilutions and kept in an incubator two hours before reading. Only negative or positive reactions at the different dilutions were recorded. A more elaborate method, such as Gardner's did not seem necessary for our limited purpose. The sera used were most kindly provided by Prof. A. Bergman, Director of the State Veterinary Bacteriological Laboratory. They were three in number, all polyvalent and obtained by injection into horses of coli and paracoli strains from calves. The results of the test are indicated in table 8.

TABLE 8

STRAIN	COLI SERUM 1							COLI SERUM 2							PARACOLI SERUM						
	1:50	1:100	1:200	1:400	1:800	1:1600	CONCENTRATION	1:50	1:100	1:200	1:400	1:800	1:1600	CONCENTRATION	1:50	1:100	1:200	1:400	1:800	1:1600	CONCENTRATION
D	+	+	+	+	+	+	0	+	+	+	+	+	+	0	+	+	+	+	+	+	0
S	+	+	0	0	0	0	0	+	+	+	0	0	0	0	+	+	0	0	0	0	0
Sd	+	+	+	+	0	0	0	+	+	+	+	+	0	0	+	+	+	+	0	0	0
Dsh	+	+	+	+	0	0	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0
Df	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dt	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Dtl	+	+	+	0	0	0	0	+	+	0	0	0	0	0	+	+	0	0	0	0	0

The only trouble experienced in making the tests was a difficulty in getting a homogeneous suspension of the pure D-strain. Fortunately this strain showed an extremely strong and rapid agglutination which made the reading possible.

Table 8 shows very strikingly that "strains" obtained from the same single cell can differ widely serologically. This is not new but was shown before by Gardner and Walker (1921) and others with *Bact. typhosum*. Immunologic disparities of spores and vegetative stages of *B. subtilis* were shown by Mellon and Anderson (1919). As to the explanation of this phenomenon Theobald Smith and Reagh (1903–04) working with hog-cholera have advanced the hypothesis that there exist two kinds of agglutinin called "body agglutinin" and "flagella agglutinin," the first

produced both by motile and non-motile forms used as antigen, the second only by motile forms. The theory has not been accepted without reservation, since several authors have shown that different antigenic factors also exist in non-motile forms. Gardner and Walker, however, say that the hypothesis is not inconsistent with their experiments and I am convinced, too, that there is some connection between motility and agglutinability. My reasons are the following. The table seems to show that the more dry, dull and cracky the colonies are, the better is the agglutination and the more shiny, humid and mucoid the more refractive it is. When the type D becomes mucoid, it becomes at the same time totally inagglutinable. A similar phenomenon, perhaps in a less marked degree, is observed with the other strains. When the S-type produces dull colonies these are composed of bacteria more agglutinable than the mother "strain."

Evidently the surface tension of the bacteria is greatly changed when surrounded by a thick layer of mucoid substance. A phenomenon due to changes in the aggregation of suspended particles cannot of course be unaffected by differences in the surface-tension of the corpuscles.

The above mentioned observation that a thorough shaking with glass-beads of a broth-culture of type D produces a considerable spontaneous agglutination is of interest in this connection.

The power of the different sera mutually to impress the bacteria also changes with the variation of the microörganisms, as the behavior of the "strain" DSh shows. The paracoli serum does not possess any chemical complexes capable of producing visible aggregation in a suspension of the variant DSh. But the coli sera A and B possess this power. The fact that the mucoid "strain" in my experiment did not show any agglutination must not be taken as a proof that such strains are impossible to agglutinate, since Gratia, using sera prepared with the same bacteria that acted as antigen, obtained agglutination of mucoid cultures too. Nor are the microörganisms of the dull opaque colonies always easy to agglutinate, as the experiments of Baerthlein and

Gratia show. Serological tests specially directed toward this point would perhaps throw more light on the question. The conclusion of the argument must be, I think, that as motility and non-motility are connected with differences on the bacterial surface, it is probable that there must be some parallelism between motility and serological conditions.

Finally I want to call attention to an article by Jacobsen (1910) describing a strain of *Bact. typhosum* as being the cause of an epidemic in Denmark. The strain grows in very tiny colonies, on agar and is very slightly agglutinable, which caused difficulties in fighting the epidemic. Jacobsen thought that the delayed growth was due to chemical substance produced by repeated sterilizing of the agar, but Müller (1911) who examined the same strain, showed that this was not the case and that the tiny growth gave rise to secondary colonies of luxuriant vegetation, which were identical with the common type of *Bact. typhosum* and highly agglutinable. Evidently the behavior of my strains DF and DFL is a phenomenon of the same kind, corresponding in every way to that described by the authors mentioned.

Before finishing this article I wish to express an opinion with regard to epidemic diseases caused by microörganisms also living as saprophytes. In a paper on the prevention of pneumonia Rufus Cole (1918) writes: "So far as pneumonia due to these types of organisms (parasitic pneumococci) is concerned, the conditions do not differ essentially from those in diseases like diphtheria or cerebro-spinal fever in which the acquiring of the infectious agent is considered of primal importance." The observations with *Bact. coli* are not inconsistent with this point of view since they teach us that the variants once they occur retain their qualities with a relative heredity which is stronger the more rapidly the microörganism is permitted to multiply. The new discoveries about bacterial variation, however, show that the problem must be regarded also from other points of view and they illuminate at least one part of the mechanism which operates when saprophytic strains change into parasitic ones. Possibly studies on bacterial variation will in the future give a clue to the solution of the question as to how an epidemic dies

out. There are features in some epidemics, for example influenza, indicating that the parasitic qualities are hereditary only for a limited time. Perhaps a struggle for life among the variants plays a great rôle in the epidemiology. Investigations of Thomsen (1921) in cerebrospinal fever point in this direction. I myself have found in several cases of *Colitis ulcerosa* pure cultures of the motile form of *Bact. coli* only. Investigations are going on in order to find which type is prevalent in cases where *Bact. coli* acts as a parasite, for example in pyelonephritis.

SUMMARY

1. In an old laboratory culture of *Bact. coli*, two types of colonies were observed, one dull dry, cracky (D), and one shining, humid, transparent (S) the former being non-motile the latter motile.

2. The D-type showed growth in broth-tubes of a pH value varying between 5.0 and 8.6. The corresponding figures for the S-type were 4.3 to 9.4. The growing bacteria readjusted both the acid and alkaline tubes to a slightly acid reaction. A too high acidity, however, killed the growing bacteria before an adjustment of the hydrogen ion concentration had occurred. The bacteria in the tubes of a hydrogen ion concentration too high or too low to permit growth were rapidly killed.

3. The high and low hydrogen ion concentration produced an increased bacterial lag and a diminution of the total amount of growth despite the readjustment of the reaction.

4. In broth-cultures of varying hydrogen ion concentration permitted to age, the D-type produced variants rapidly, the S-type slowly. Some of the D-variants resembled in every respect except motility the S-type and conversely the S-variants resembled the D-type. On agar-plates the variations were visible as secondary colonies.

5. The variants could be kept pure for months by repeated transfers from broth to broth or on agar plates. When left in broth-tubes they produced colonies of the mother type and new variants.

6. The D-type was agglutinated at high, the S-type at a very low dilution by polyvalent coli serum. The shining variants of

the D-type were less agglutinable than the mother type and the full variants of the S-type conversely more agglutinable. One extremely mucoid variant of the D-type had totally lost its agglutinability. This phenomenon seems to indicate that the variation in agglutinability is due to changes in the mucoid layer surrounding the bacteria. A broth-culture thoroughly shaken with glass-beads showed spontaneous agglutination.

7. One variant of the D-type showing a very tiny and barely visible growth and a bad agglutination produced a luxuriant secondary vegetation of another type agglutinable at higher titer. This phenomenon corresponds to the observations of Jacobsen and Müller with *Bact. typhosum*. The secondary vegetation sometimes formed beautiful rings round the tiny almost invisible original central growth.

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PLATE 1

FIG. 1. A one-month-old agar plate of the S-type showing a few secondary colonies forming rings.

FIG. 2. An agar plate of the D-type one month old demonstrating secondary colonies in the form of droplets.

FIG. 3. A three-weeks-old slanted agar culture of the type DT containing two colonies with an initial tiny growth and a secondary luxuriant vegetation, forming in one of the colonies a surrounding ring, in the other a ring and a central heap.

FIG. 4. An agar plate two weeks old seeded with a sample removed from the 7th tube in the series of the D-type on March 5th and containing not only several big colonies of the dull type with secondary droplets but also small colonies of a variant resembling the S-type. Fishing is made from both types and the needle has cracked the dull, dry, big, colony. From the small ones, on the contrary humid masses have been dragged out, only owing to the paste-like consistency of these colonies.

FIG. 5. Two kinds of colonies obtained by fishing from the secondary colonies on the agar plate indicated in fig. 1. The dark ones are the variants, the others resembling the mother type in every respect.

FIG. 6 AND 7. Colonies on three-weeks-old agar plates at different magnification of the type DT. The tiny transparent growth is seen in the periphery of the irregularly denoted colonies, the central parts being occupied by a luxuriant secondary vegetation.

FIG. 8. A ten-days-old agar plate of the DF-type. The mucoid, fluid parts of the vegetation are mostly dried up. The non-mucoid growth shows secondary colonies of the same sort as in fig. 2.

PLATE 2

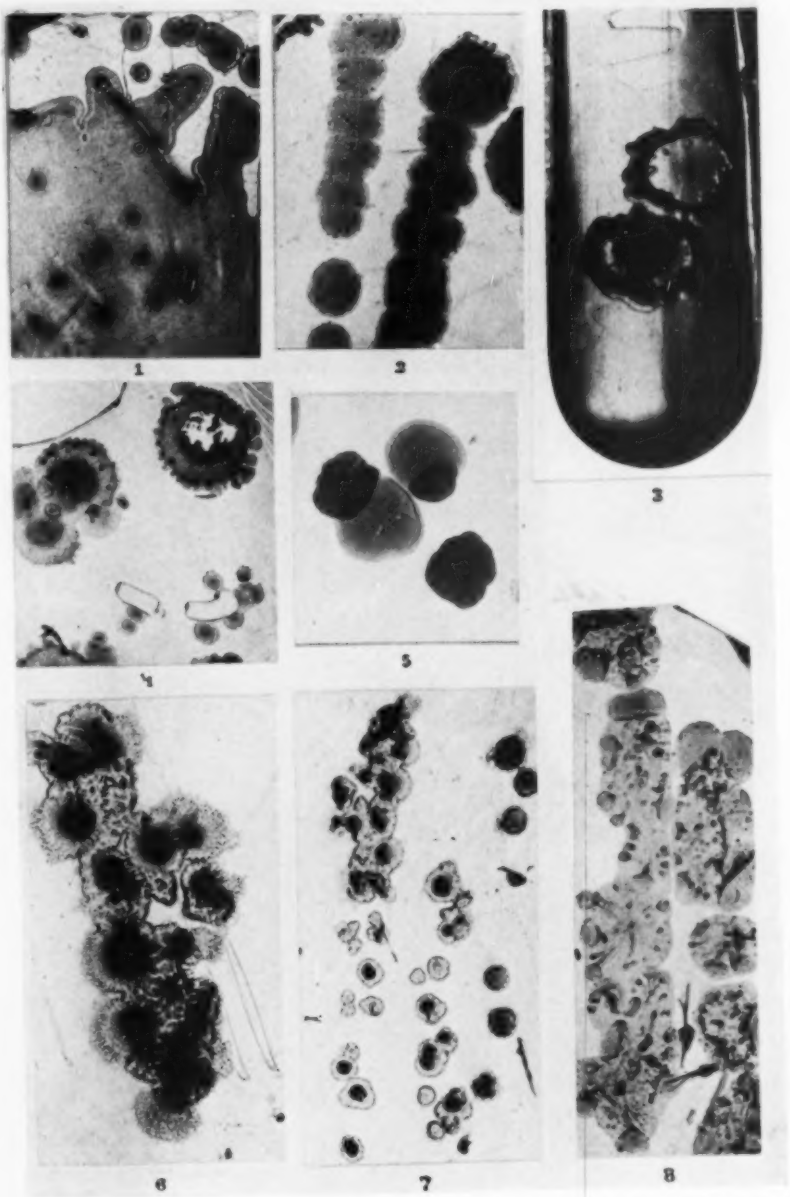
FIG. 9. Mucoid growth on the border of colonies of the dull type. The plate is two weeks old.

FIG. 10. Two broth-cultures of the series seeded on February 13 being two months old. The D-type to the left, the S-type to the right.

FIG. 11. Mucoid growth dissolving the dry colonies.

FIG. 12. Colonies obtained by fishing from the dark colonies demonstrated on figure. 5. The ring forms are a transient variant, the others a very stable one of a type resembling the D-type and signed SD.

FIG. 13. Another part of the plate explained in figure 8.



(Bergstrand: Variation of Bacterium coli)

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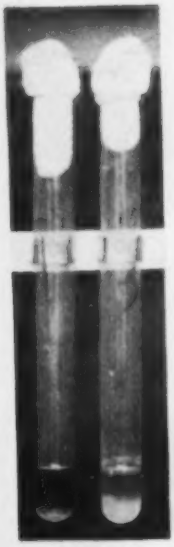

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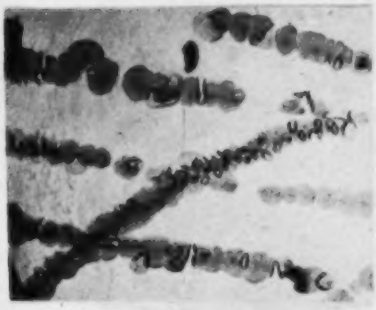

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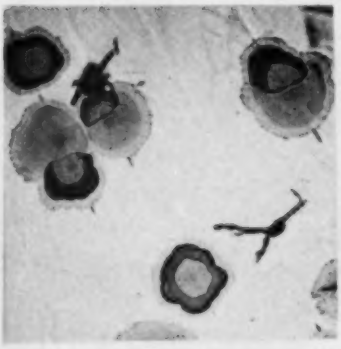

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(Bergstrand: Variation of Bacterium coli)